

# World Journal of *Methodology*

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## New developments and controversies in iron metabolism and iron chelation therapy

Christina N Kontoghiorghe, George J Kontoghiorghe

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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; Kontoghiorghe 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/deferrioxamine 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 The uniform distribution of iron in iron overload between organs and within each organ is no longer valid.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

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**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.

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## 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<sup>[1,2]</sup>. 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<sup>[3]</sup>.

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 constraints 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<sup>[4,5]</sup>.

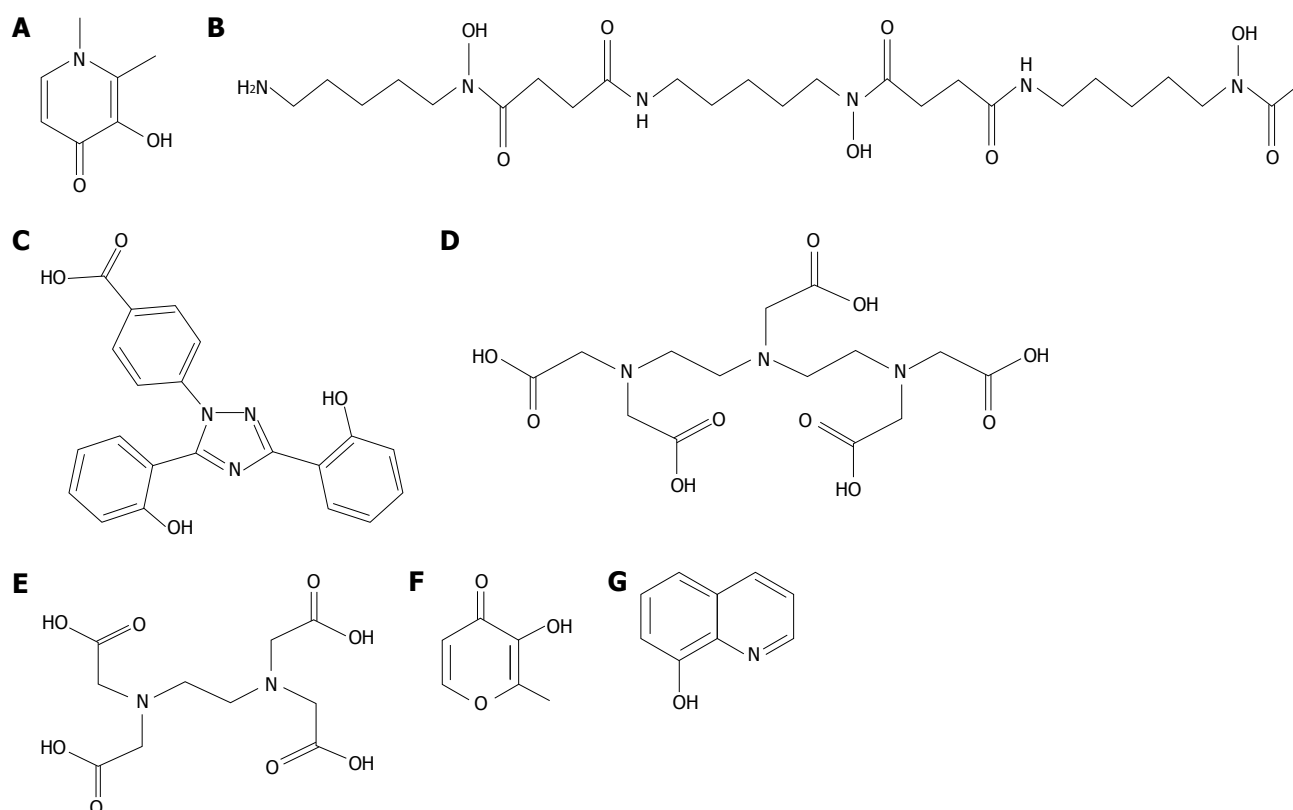
The disease with the highest mortality and morbidity rate related to iron metabolic disorders worldwide is thalassaemia, which is found mainly in developing countries of South East Asia, Middle East and Mediterranean. More than 100000 thalassaemia babies are born every year with 9000 in India alone, most dying without treatment<sup>[4-6]</sup>. 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<sup>[5]</sup>. 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<sup>[7,8]</sup>. 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)<sup>[9]</sup>.

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<sup>[5]</sup>. 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<sup>[5]</sup>.

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<sup>[10,11]</sup>.

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<sup>[1,2]</sup>. 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<sup>[5]</sup>.

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<sup>[12,13]</sup>. 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 morbidity 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<sup>[5]</sup>. 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<sup>[14]</sup>. It can also sometimes be found in the Fe (IV) form in haem, which is associated with pathological effects<sup>[15]</sup>.

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<sup>[1,2,14]</sup>. 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<sup>[1,2,16,17]</sup>.

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<sup>[3,18]</sup>. Such biomolecular damage can lead to subcellular, cellular, tissue and organ damage, which can be permanent or reversible<sup>[19]</sup>. 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<sup>[20]</sup>.

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<sup>[19]</sup>. However, at high concentrations excess iron can cause permanent damage and can be fatal, *e.g.*, in cardiac iron overload in thalassaemia<sup>[7,8]</sup>.

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<sup>[1,2,16]</sup>.

Iron absorption, transport, storage, utilisation, recycling and excretion are mostly genetically controlled by effective regulatory metabolic pathways, homeostatic mechanisms and related proteins<sup>[1,2]</sup>. 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.*<sup>[1,2,14,19]</sup>. 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<sup>[1,2,14,19]</sup>. 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<sup>[1,2,14,19]</sup>.

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<sup>[1,2,21]</sup>. 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<sup>[22,23]</sup>. 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<sup>[24]</sup>. 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<sup>[25]</sup>. 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<sup>[25]</sup>. 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<sup>[26,27]</sup>. Iron overload can also be caused by regular red blood cell transfusions in conditions such as in thalassaemia, myelodysplasia and sickle cell diseases<sup>[7,8,28]</sup>. 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<sup>[29,30]</sup>.

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<sup>[10,11,19]</sup>.

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<sup>[27]</sup>. 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*<sup>[31,32]</sup>.

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*<sup>[19,33-35]</sup>.

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

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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<sup>[14]</sup>.

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*<sup>[14,18,36]</sup>. Similarly, the presence of other metals may interfere with chelator iron binding and chelators may affect the metabolic pathways of other metals<sup>[37,38]</sup>. Overall many interactions can affect the efficacy and toxicity of the chelating drugs *in vivo*<sup>[14,36,37]</sup>.

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 lipophilic 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, <i>e.g.</i> , 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 <i>in vitro</i> , <i>in vivo</i> 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)<sup>[14,36]</sup>.

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<sup>[5,7,8]</sup>.

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)<sup>[5,7,8]</sup>.

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<sup>[5,9,39-41]</sup>.

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<sup>[9,42]</sup>. In contrast, high morbidity and mortality have been reported in different categories of patients that have been treated with DFRA<sup>[43,44]</sup>. The efficacy of iron removal from thalassaemia patients by DFRA is lower than DF or L1, especially regarding iron removal from the heart<sup>[45]</sup>. The most effective treatment of cardiac iron overload are selected combinations of L1 and DF<sup>[46]</sup>.

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<sup>[45]</sup>. 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<sup>[47]</sup>. In most countries the selection of the chelating drug for the treatment of iron loaded patients depends on the commercial influence of pharmaceutical

companies<sup>[5]</sup>. 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<sup>[5]</sup>.

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<sup>[48-50]</sup>. 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<sup>[9]</sup>.

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<sup>[9,36,51]</sup>.

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<sup>[10,11,19]</sup>. 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*<sup>[10,11,19]</sup>. As in many other cases of drug development the introduction prospects of L1 in these diseases is based on commercial and not ethical criteria<sup>[5]</sup>.

## 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*<sup>[48-50]</sup>. 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<sup>[52,53]</sup>.

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.

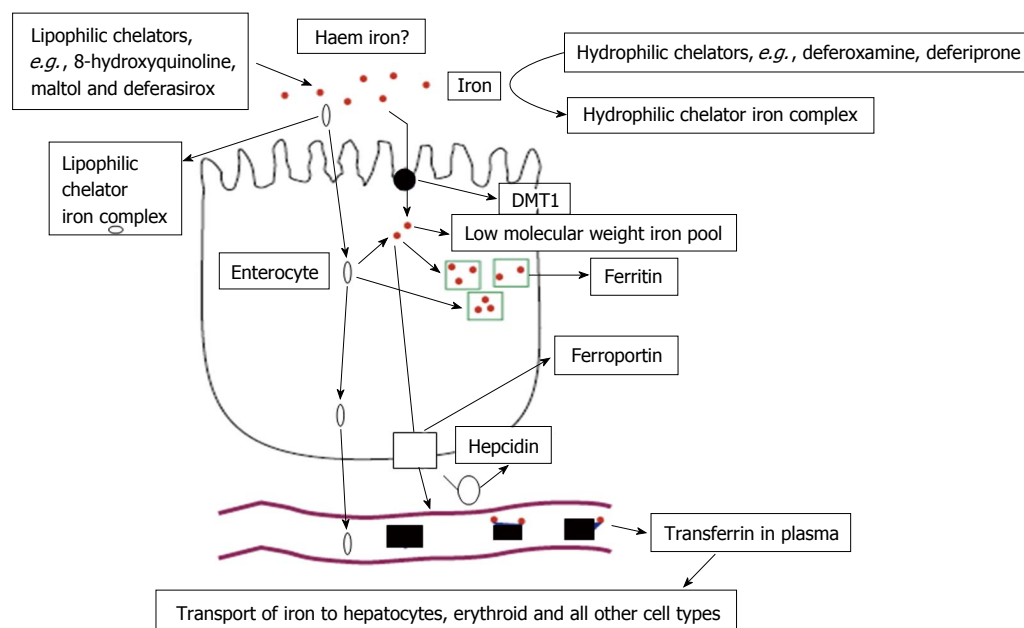
Variations in the progression of neurodegeneration, cardiomyopathy and other toxic side effects observed among Friedreich Ataxia patients is thought to be related to the production of the protein frataxin and many other factors influencing the rate of accumulation and toxicity of iron in mitochondria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

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<sup>[14,36]</sup>.

## 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)<sup>[1,2]</sup>.

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)<sup>[22]</sup>. 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<sup>[25,29,30]</sup>. 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<sup>[22,25]</sup>. 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<sup>[25,26]</sup>.

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)<sup>[22,25,26]</sup>. 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)<sup>[22]</sup>. 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)<sup>[56,57]</sup>. Maltol in particular, was originally identified as a chelator intended for clinical use in iron deficiency at the same time that L1 was identified for the treatment of iron overload<sup>[58]</sup>. 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<sup>[59,60]</sup>.

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<sup>[22,57]</sup>. 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<sup>[61]</sup>. It is envisaged that overall many naturally occurring dietary compounds and medicinal drugs with chelating properties will affect iron absorption in a manner similar to that observed by lipophilic and hydrophilic chelators<sup>[18,25]</sup>.

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 ceruloplasmin 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<sup>[62,63]</sup>.

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<sup>[64]</sup>. 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<sup>[62,63,65,66]</sup>. Almost all thalassaemia patients with serum ferritin greater than 500 µg/L appear to have fully saturated transferrin and different amounts of NTBI<sup>[62,63,65]</sup>. 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<sup>[48-50,67]</sup>.

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<sup>[68,69]</sup>.

## 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<sup>[25,70]</sup>. 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<sup>[25,70]</sup>.

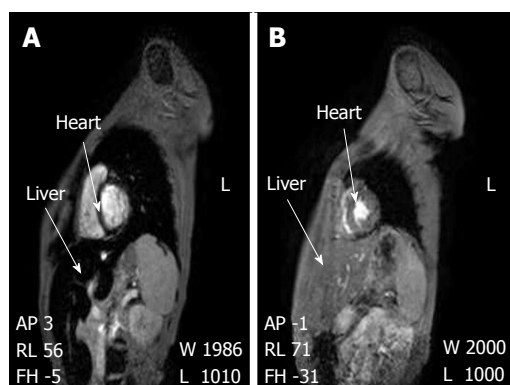
In general several factors such as the body iron load, plasma iron concentration, physical activity, infections, pathological conditions and dietary habits affect the level of iron excretion<sup>[25]</sup>. The presence of regulatory iron excretion is also supported by other clinical findings such as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

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<sup>[73]</sup>. 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<sup>[74]</sup>. 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<sup>[14,25,47]</sup>. 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)<sup>[14,25]</sup>.

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<sup>[45,46,50,74,75]</sup>. 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<sup>[74]</sup>.

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)<sup>[76-80]</sup>. 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<sup>[77]</sup>. Thereafter monotherapy of L1 at 50-100 mg/kg per day is sufficient in most cases for maintaining normal range body iron store levels<sup>[78,80]</sup>.

Many naturally occurring 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 deferioxamine 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  $\mu\text{g/L}$ , 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  $\mu\text{g/L}$ , 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 occurring 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<sup>[25]</sup>. The chelating efficacy of naturally occurring 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*<sup>[58,81]</sup>. The reaction is L1 concentration dependent and partial transit de-ironing from transferrin is observed in the serum of iron loaded thalassaemia patients<sup>[58,62,63,81-84]</sup>.

In the intracellular iron mobilisation by chelators, the transit low molecular weight iron pool is readily available followed by haemosiderin and then ferritin iron<sup>[85]</sup>. The reaction is chelator concentration dependent and takes 2-3 d to reach completion<sup>[86]</sup>. 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<sup>[87]</sup>. It appears that there is lower exposure of the surface iron core to chelators by comparison to larger iron cores<sup>[87]</sup>. 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<sup>[85,87]</sup>.

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

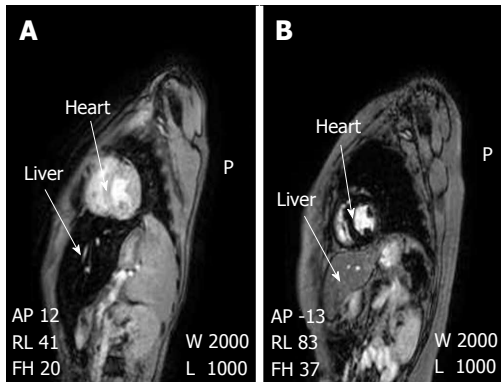
## CONDITIONS WITH ABNORMAL IRON DEPOSITION AND THE RELOCATION OF IRON BY CHELATORS

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 Kupffer 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<sup>[50,90-93]</sup>. 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)<sup>[14,90,93]</sup>. 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<sup>[94,95]</sup>.

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)<sup>[96,97]</sup>. 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<sup>[95]</sup>. Furthermore, following splenectomy excess iron may be diverted to the heart causing myocardial iron loading and cardiomyopathy<sup>[97]</sup>.

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<sup>[93]</sup>. 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<sup>[50]</sup>. 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  $T2^* \geq 6.3$ )] 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<sup>[50]</sup>. 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<sup>[98,99]</sup>. 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<sup>[1,2,98,99]</sup>.

A similar mechanism of increased hepcidin production leading to plasma iron reduction is thought to operate in the hypoferraemia of infectious diseases. This mechanism appears to reduce transferrin bound iron and iron bioavailability to the siderophores of microbes restricting their growth<sup>[100,101]</sup>. 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<sup>[35,102]</sup>. A hepcidin independent pathway for the hypoferraemia in infections has also

been identified<sup>[103]</sup>. Within this context pharmacologic modulation of iron metabolism and chelation therapy may be potential strategies to control infection<sup>[35,63]</sup>.

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<sup>[54,55,95,104-106]</sup>. 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<sup>[9,107-109]</sup>.

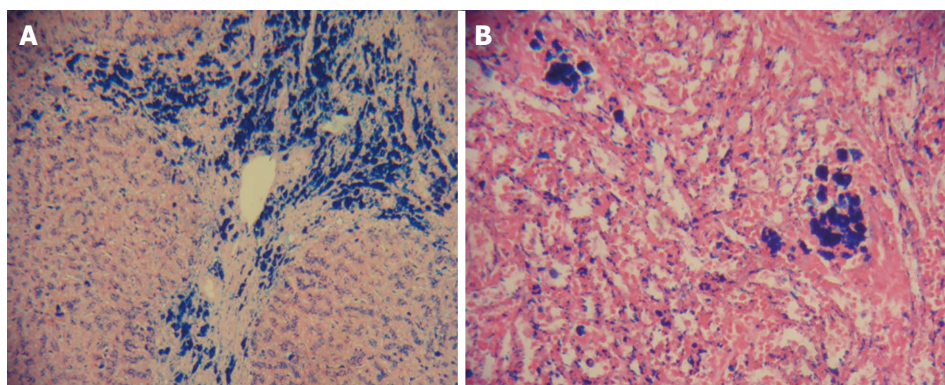
The localisation of focal deposited iron in the brain has been recently identified by MRI in many neurodegenerative and other diseases such as Friedreich Ataxia, Parkinson's and Alzheimer's diseases and Hallevorden-Spatz syndrome<sup>[110-115]</sup>. However, a major difference between the above conditions 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  $2 \times 2$  g/d for a week. A substantial increase in haemoglobin levels were observed at the end of the study<sup>[116,117]</sup>. 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<sup>[118]</sup>. 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<sup>[83,119]</sup>. 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<sup>[116,117]</sup>.

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 ( $\times 20$ ) of a 29-year-old, 55 kg male thalassaemia patient. The liver biopsy was obtained during splenectomy. A: Liver section showing non uniform 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 cytoplasm 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  $\mu\text{g/L}$ . 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<sup>[120]</sup>. Similarly, neurological and heart function benefits were identified in further L1 trials in Friedreich Ataxia and other patients<sup>[121-124]</sup>.

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<sup>[125]</sup>. 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<sup>[125]</sup>.

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<sup>[9,14,19,22,63]</sup>. However, many therapeutic developments are almost exclusively based on commercial and not ethical considerations<sup>[5,126-130]</sup>. 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<sup>[5,131-134]</sup>. 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<sup>[5,131-135]</sup>.

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<sup>[135]</sup>. 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<sup>[5,131-135]</sup>. 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*<sup>[5,131-135]</sup>. 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<sup>[5]</sup>.

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<sup>[5,43,44,136-140]</sup>. 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 assessment of the use of DFRA in thalassaemia intermedia instead of L1 or DF<sup>[22,138]</sup>. Furthermore, many clinical investigators have also questioned the therapeutic benefits from use of DFRA or of other chelating drugs in myelodysplasia and sickle cell anaemia patients<sup>[141,142]</sup>.

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*<sup>[43,44,143,144]</sup>. 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<sup>[73]</sup>. 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<sup>[5,36]</sup>.

Commercial and academic conflicts in relation to L1 development are widely published in the medical literature since its discovery<sup>[5]</sup>. 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<sup>[5]</sup>. 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<sup>[5]</sup>.

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<sup>[5]</sup>. 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<sup>[15,145,146]</sup>. 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<sup>[5,145,146]</sup>.

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<sup>[5]</sup>. 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<sup>[45,46,65,67]</sup>. This issue partly diverts attention from the difference in the ability of chelating drugs in the mobilisation of iron from the heart<sup>[12]</sup>. 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<sup>[147-149]</sup>.

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<sup>[150-152]</sup>. 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<sup>[153]</sup>. 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)<sup>[153-155]</sup>. It is only recently that the health authorities in the United States took an interest on its therapeutic properties in cardiovascular and other conditions<sup>[155,156]</sup>.

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<sup>[19,157]</sup>. 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<sup>[158]</sup>.

## 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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## 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.

graphical (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

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**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.

Sharma P, Das R. Cation-exchange high-performance liquid chromatography for variant hemoglobins and HbF/A2: What must hematopathologists know about methodology? *World J Methodol* 2016; 6(1): 20-24 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i1/20.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i1.20>

## 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<sup>[1]</sup>. 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<sup>[1,2]</sup>. 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<sup>2</sup>) 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<sup>[3]</sup>. 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<sup>[3,4]</sup>. 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<sup>[5]</sup>. 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<sup>[3,6]</sup>.

## 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<sup>[6]</sup>.

### 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

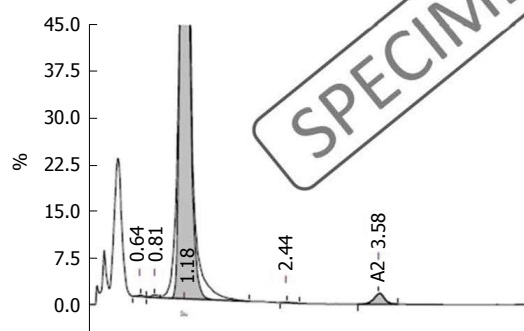
**io-Rad CDM System****DM 5.1 VII TURBO Instrument****PATIENT REPORT****V2\_BThal**

Patient data	Analysis data
Sample ID: 23287-15	Analysis performed: 01/04/2015 14:05:36
Patient ID:	Injection number: 1436R
Name:	Run number: 48
Physician:	Rack ID: 0005
Sex:	Tube number: 8
Dob:	Report generated: 07/04/2015 17:06:41
Comments:	Operator ID:

Peak name	Calibrated area (%)	Area (%)	Retention time (min)	Peak area
Unknown	-	0.1	0.64	2209
P1	-	0.3	0.81	5135
F	100.5 <sup>1</sup>	-	1.18	1537546
A0	-	0.1	2.44	1104
A2	1.8 <sup>1</sup>	-	3.58	30235

F concentration = 100.5%<sup>1</sup>A2 concentration = 1.8%<sup>1</sup>

Total area: 1576229

<sup>1</sup>Values outside of expected ranges**Analysis comments**

**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  $\beta$ -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.



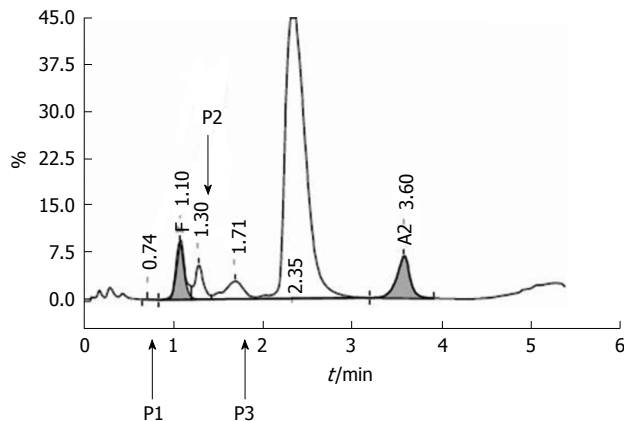
Peak name	Area (%)	Retention time (min)	Peak area	Old CF	New CF
P1	-	0.74	655	-	-
F	6.3	1.10	170743	1.004	1.025
P2	-	1.30	102589	-	-
P3	-	1.71	123335	-	-
A0	-	2.35	2090989	-	-
A2	7.5	3.60	203063	0.887	0.901

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  $\beta$ -thalassemia trait with increased HbF (HbF levels between 1%-5% are found in approximately 30% of  $\beta$ -thalassemia trait cases, and occasional ones may have even higher levels). Calibrator data had shown above.

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  $\times 100 \div$  total area (*i.e.*,  $1537546 \times 100 \div 1576229 = 97.5\%$ ).

### 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  $\leq 1\%$  of total area<sup>[6]</sup>. Unknown peaks at longer retention times and those  $> 1\%$  should be paid greater attention as transfusion-transmitted peaks, HbA2' may present as small peaks<sup>[7]</sup>. 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.

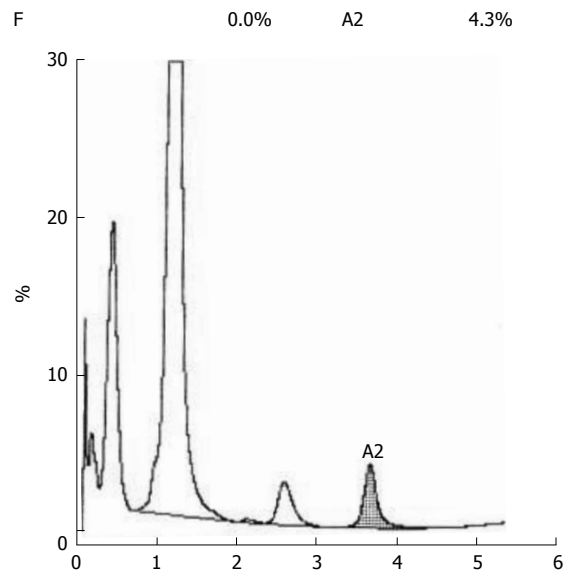
### Pre-integration peaks

These peaks, with retention time  $< 1$  min, are not

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



**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.

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%<sup>[1,3,4]</sup>. 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  $3.8 \pm 0.7$  and  $4.3 \pm 0.4$  respectively (unpublished data). P2 is comprised of glycated hemoglobin and levels  $\geq 6.5\%$  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  $\alpha$ -globin chain variant). It is also elevated in cases with the HbE variant (that elutes in the HbA2 window) and in aged specimens<sup>[1,3,4]</sup>. 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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## 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 demon-

strated 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

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**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.

Gong J, Sachdev E, Mita AC, Mita MM. Clinical development of reovirus for cancer therapy: An oncolytic virus with immune-mediated antitumor activity. *World J Methodol* 2016; 6(1): 25-42 Available from: URL: <http://www.wjgnet.com/2222-0682/full/>

## 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<sup>[1,2]</sup>. 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)<sup>[3]</sup>. However, when symptomatic, reovirus infection is characterized by mild enteric and respiratory symptoms in humans<sup>[1-5]</sup>. Wild-type reovirus is ubiquitous throughout the environment with seropositivity having been documented in as many as 70%-100% of subjects<sup>[3]</sup>. 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<sup>[2,3,5]</sup>.

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)<sup>[1,2,4-7]</sup>. 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<sup>[8,9]</sup>. 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<sup>[10,11]</sup>. 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<sup>[12,13]</sup>.

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*/Raf/MEK/p38 pathway in promoting preferential reovirus replication<sup>[14,15]</sup>. 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 2 $\alpha$  (eIF-2 $\alpha$ ), 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<sup>[13,16]</sup>. 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<sup>[17-19]</sup>.

## 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<sup>[20]</sup>. 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<sup>[20]</sup>. 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<sup>[21]</sup>. 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*<sup>[22]</sup>. 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<sup>[23]</sup>.

The incidence of activated *Ras* mutations in colon cancer is approximately 50%<sup>[15]</sup>. 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<sup>[24]</sup>. 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<sup>[25]</sup>. 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 subviral particles (ISVPs) and proper localization and quantity of junctional adhesion molecule-1 on tumor cells for productive lysis<sup>[26-28]</sup>. Furthermore, colon cancer cell lines HEK293 and HCT116 demonstrated sensitization to reovirus-induced apoptosis by downregulation of nuclear

factor-kappa B (NF- $\kappa$ B) through inhibition of glycogen synthase kinase-3 $\beta$ <sup>[29]</sup>.

In adenocarcinomas of the pancreas, the incidence of K-Ras mutations is among the highest in human cancer (approximately 90%)<sup>[15]</sup>. 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*<sup>[30]</sup>. 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<sup>[30]</sup>. Administration of reovirus also induced regression in immunocompetent hamster models of pancreatic cancer with liver and peritoneal metastases compared to controls<sup>[31,32]</sup>.

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<sup>[15,33]</sup>. 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*<sup>[33]</sup>. Additionally, intravesical administration of reovirus resulted in significantly higher tumor-free survival in an orthotopic rat model of bladder cancer compared to control<sup>[34]</sup>. 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<sup>[15]</sup>. 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)<sup>[35]</sup>.

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<sup>[3-5,36]</sup>. 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<sup>[36-39]</sup>. Furthermore, the presence of replicating reovirus was confirmed in *ex vivo* surgical breast cancer specimens<sup>[36]</sup>. 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<sup>[37]</sup>. 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<sup>[4,5,25]</sup>. 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<sup>[25]</sup>. All 3 *ex vivo* human ovarian tumor surgical biopsy specimens also demonstrated susceptibility to reovirus infection<sup>[25]</sup>.

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<sup>[40-42]</sup>. Intriguingly, although reovirus-induced cytotoxicity was observed in several head and neck carcinoma cell lines, correlative analyses revealed no associations between phosphorylated eIF-2 $\alpha$  or EGFR levels and cytopathic effects suggesting that reovirus oncolysis appears to occur independently of PKR, Ras signaling, and EGFR signaling pathways<sup>[43,44]</sup>.

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)<sup>[15,45]</sup>. 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<sup>[3,45]</sup>. 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<sup>[45]</sup>. 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<sup>[45]</sup>.

Treatment of acute myeloid leukemia (AML) with reovirus showed marked antitumor responses in 2 out of 4 AML cell lines *in vitro* and in 8 out of 10 peripheral blood primary AML specimens *ex vivo*<sup>[46]</sup>. 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 heterogeneous response to reovirus that correlated with levels of activated Ras and proteolytic disassembly of reovirus into ISVPs *in vitro*<sup>[47]</sup>. The discrepancies in sensitivity to reovirus infection between various hematologic malignancies have been attributed, in part, to differential Ras activation and interferon sensitivities<sup>[4,5,45,47]</sup>. 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<sup>[48]</sup>. 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*<sup>[48]</sup>. 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<sup>[49]</sup>. 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<sup>[50,51]</sup>.

### 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<sup>[52-54]</sup>. 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*<sup>[55]</sup>. 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  $\times$  5 fractions], respectively<sup>[40]</sup>.

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<sup>[56,57]</sup>. 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<sup>[58]</sup>. Although reovirus alone demonstrated potent cytotoxicity in 7 of 9 non-small cell lung cancer (NSCLC) cell lines *in vitro*, heterogeneous synergistic effects on cell killing were observed with reovirus in combination with cisplatin, gemcitabine, or vinblastine on NSCLC cancer cell lines *in vitro*<sup>[59]</sup>. The reovirus and paclitaxel combination, however, showed synergistic cell killing in all NSCLC cell lines *in vitro* characterized by enhanced apoptosis<sup>[59]</sup>.

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<sup>[60]</sup>. 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<sup>[60]</sup>. 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<sup>[60]</sup>.

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<sup>[27,61-64]</sup>. Reovirus and docetaxel also produced significant tumor growth retardation in a murine prostate cancer xenograft<sup>[63]</sup>. 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<sup>[65]</sup>. 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<sup>[65]</sup>.

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<sup>[1,5,6,66]</sup>. 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<sup>®</sup>, developed by Oncolytics Biotech Inc. (Calgary, Canada)<sup>[2-4,67]</sup>. 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<sup>[68]</sup>. In a dose-escalation design, doses of  $1 \times 10^7$  plaque forming units (PFU) once weekly up to maximum doses of  $1 \times 10^{10}$  PFU once weekly were used<sup>[68]</sup>. Out of 19 patients, dose-limiting toxicities (DLTs) and a maximum-tolerated dose (MTD) were not observed even at maximum dose<sup>[68]</sup>. 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<sup>[68]</sup>. The best overall response  $\geq 6$  wk was complete response (CR) in 1 (5.3%), partial response (PR) in 2 (10.5%), stable disease (SD) in (21.1%)<sup>[68]</sup>.

REO 002 enrolled 6 patients with localized prostate cancer who received a single intratumoral injection of  $1 \times 10^7$  PFU of reovirus 3 wk prior to planned prostatectomy as definitive cancer treatment<sup>[42]</sup>. 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<sup>[42]</sup>. 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<sup>[42]</sup>.

Another phase I study (REO 003) involved single stereotactic intralesional injection of reovirus at doses ranging from  $1 \times 10^7$  tissue culture infectious dose-50 (TCID<sub>50</sub>) to  $1 \times 10^9$  TCID<sub>50</sub> in 12 patients with progressive or recurrent malignant gliomas<sup>[69]</sup>. 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  $\gamma$ -glutamyl transpeptidase<sup>[69]</sup>. 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<sup>[69]</sup>. 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<sup>[70]</sup>. 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<sup>[70]</sup>. Additionally, only one of these three grade 3 AEs was possibly related to infusion of reovirus<sup>[70]</sup>. During the study period of 24 wk, ten patients were reported to have stable disease, four with progressive disease, and one with partial response<sup>[70]</sup>. 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)<sup>[70]</sup>. The one patient that did achieve a partial response did receive the maximum dose<sup>[70]</sup>.

REO 004 included 18 patients with advanced solid tumors treated with intravenous (IV) reovirus from  $1 \times 10^8$  TCID<sub>50</sub> to  $3 \times 10^{10}$  TCID<sub>50</sub> 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<sup>[71]</sup>. No DLTs were observed and the most common AEs included myalgia, fatigue, and fever<sup>[71]</sup>. 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)<sup>[71]</sup>. 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<sup>[71]</sup>. One phase I study (REO 005) pitted IV reovirus against various refractory or metastatic cancers, and a MTD was reached at a dose of  $3 \times 10^{10}$  TCID<sub>50</sub> 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)<sup>[72]</sup>. No DLTs were observed and the most common AEs were fever, fatigue, and headache<sup>[72]</sup>. 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<sup>[72]</sup>.

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  $1 \times 10^8$  TCID<sub>50</sub> to  $1 \times 10^{10}$  TCID<sub>50</sub> intratumoral injection of reovirus on days 2 and 4 with 20 Gy local irradiation daily  $\times$  5 fractions while phase 1b treated patients with  $1 \times 10^{10}$  TCID<sub>50</sub> intratumoral injection of reovirus twice weekly for 1-3 wk with 36 Gy local irradiation  $\times$  12 fractions over 16 d<sup>[73]</sup>. 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	$1 \times 10^7$ PFU to $1 \times 10^{10}$ PFU intralesional injection once or $3 \times$ weekly (dose escalation)	Out of 19 patients, best overall response $\geq 6$ 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	$1 \times 10^7$ 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	$1 \times 10^7$ TCID <sub>50</sub> to $1 \times 10^9$ TCID <sub>50</sub> 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 $1 \times 10^8$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> 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 $1 \times 10^8$ TCID <sub>50</sub> once every 28 d to $3 \times 10^{10}$ TCID <sub>50</sub> once daily for 5 d every 28 d (dose escalation); IV reovirus $3 \times 10^{10}$ TCID <sub>50</sub> 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	$1 \times 10^8$ TCID <sub>50</sub> to $1 \times 10^{10}$ TCID <sub>50</sub> intratumoral injection on days 2 and 4 with 20 Gy local irradiation daily $\times 5$ fractions vs phase 1b: $1 \times 10^{10}$ TCID <sub>50</sub> intratumoral injection twice weekly from 1-3 wk with 36 Gy local irradiation $\times 12$ fractions over 16 d (two-stage dose escalation); intratumoral $3 \times 10^{10}$ TCID <sub>50</sub> $\times 2$ injections with 20 Gy $\times 5$ fractions and intratumoral $1 \times 10^{10}$ TCID <sub>50</sub> $\times 6$ injections with 36 Gy $\times 12$ 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 $1 \times 10^8$ TCID <sub>50</sub> to $1 \times 10^{10}$ TCID <sub>50</sub> (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 $1 \times 10^9$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> on day 1 (dose escalation) with 30-min IV infusion of gemcitabine 1000 mg/m <sup>2</sup> days 1 and 8 every 21 d ( $1 \times 10^{10}$ TCID <sub>50</sub> 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 $3 \times 10^9$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 (dose escalation) with 60-min IV infusion of docetaxel 75 mg/m <sup>2</sup> day 1 every 21 d ( $3 \times 10^{10}$ TCID <sub>50</sub> reovirus days 1-5 every 21 d became recommended phase II dose with docetaxel)	Out of 16 patients, best overall response was PR $\geq 2$ 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 $\geq 2$ 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 $1 \times 10^{10}$ TCID <sub>50</sub> daily $\times 5$ 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 $1 \times 10^{10}$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 every 28 d (dose escalation) with standard FOLFIRI doses (recommended phase II dose was irinotecan 150 mg/m <sup>2</sup> with $3 \times 10^{10}$ TCID <sub>50</sub> 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 $3 \times 10^9$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> 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<sub>50</sub>: 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 $3 \times 10^9$ TCID <sub>50</sub> to $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 (dose escalation) with IV paclitaxel 175 mg/m <sup>2</sup> over 3 h and IV carboplatin AUC <sub>5</sub> (over 30 min) on day 1 every 21 d ( $3 \times 10^{10}$ TCID <sub>50</sub> 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 $3 \times 10^{10}$ TCID <sub>50</sub> 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: $1 \times 10^{10}$ TCID <sub>50</sub> intratumoral injection on days 2 and 4 with 4 Gy local irradiation daily $\times 5$ (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 $3 \times 10^{10}$ TCID <sub>50</sub> 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 $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 every 28 d	Out of 53 enrolled patients, best overall response was SD $\geq 12$ 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 $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 with IV paclitaxel 175 mg/m <sup>2</sup> over 3 h and IV carboplatin AUC <sub>5</sub> (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 $\geq 12$ wk for a CBR of 46%
II (REO 016)	Recurrent or metastatic NSCLC	60-min IV infusion $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 with IV paclitaxel 175 mg/m <sup>2</sup> over 3 h and IV carboplatin AUC <sub>5</sub> (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 $1 \times 10^{10}$ TCID <sub>50</sub> on days 1, 2, 8 and 9 with IV infusion of gemcitabine 800 mg/m <sup>2</sup> 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 $3 \times 10^{10}$ TCID <sub>50</sub> days 1-5 with IV paclitaxel 200 mg/m <sup>2</sup> over 3 h and IV carboplatin AUC <sub>5</sub> 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 $\geq 6$ mo (33.3%)
III (REO 018)	Advanced or metastatic head and neck cancer	Randomized, double-arm, double-blinded, multicenter: 60-min IV infusion $3 \times 10^{10}$ TCID <sub>50</sub> 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<sub>50</sub>: Tissue culture infectious dose-50; AUC<sub>5/6</sub>: 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%)<sup>[73]</sup>. The best overall

response was PR in 2 patients and SD in 5 patients (out of 7 patients in phase I a) and PR in 5 patients and



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

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  $3 \times 10^9$  TCID<sub>50</sub> to  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 of IV reovirus (the last being the recommended phase II dose with docetaxel) with IV docetaxel 75 mg/m<sup>2</sup> on day 1 every 21 d<sup>[75]</sup>. 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<sup>[75]</sup>. Out of 16 patients, the best overall response was PR  $\geq$  2 cycles in 4 patients and SD  $\geq$  2 cycles in 10 patients for a CBR of 88%<sup>[75]</sup>. REO 013 enrolled 10 patients with metastatic colorectal cancer to the liver to be treated with  $1 \times 10^{10}$  TCID<sub>50</sub> of IV reovirus daily  $\times$  5 d between 6-28 d prior to planned radical resection of liver metastases<sup>[76]</sup>. There were no grade 3 or higher toxicities and the most common AEs were flu-like symptoms<sup>[76]</sup>. Resected tumor specimens from 9 patients showed staining for reovirus protein greatest in tumor when compared to surrounding tumor stroma and normal liver<sup>[76]</sup>.

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  $1 \times 10^{10}$  TCID<sub>50</sub> to  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 every 28 d with standard FOLFIRI<sup>[77]</sup>. Irinotecan 150 mg/m<sup>2</sup> with  $3 \times 10^{10}$  TCID<sub>50</sub> IV reovirus days 1-5 every 28 d

became the recommended phase II dose<sup>[77]</sup>. The most common (> 10%) grade 3 or higher toxicities were neutropenia, anemia, and thrombocytopenia, and DLTs of neutropenia were observed<sup>[77]</sup>.

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  $3 \times 10^9$  TCID<sub>50</sub> to  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 every 28 d<sup>[78]</sup>. A MTD was not reached, no DLTs were observed, and grade 3 toxicities included neutropenia, leukopenia, thrombocytopenia, and hypophosphatemia (Table 1)<sup>[78]</sup>. 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  $3 \times 10^9$  TCID<sub>50</sub> to  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 (the latter being the recommended phase II dose) with IV paclitaxel 175 mg/m<sup>2</sup> over 3 h and IV carboplatin area under curve-5 (AUC<sub>5</sub>) over 30 min on day 1 every 21 d in untreatable, relapsed, or metastatic solid cancers<sup>[79]</sup>. 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<sup>[79]</sup>. 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<sup>[79]</sup>. 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)<sup>[79]</sup>. Of the 24 patients with head and neck cancer, the median OS was 7.1 mo (CI: 4.2-11.5 mo)<sup>[79]</sup>. 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  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 with dose-escalation of daily intraperitoneal (IP) reovirus every 28 d (Table 2)<sup>[80,81]</sup>.

### 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<sup>[68,82]</sup>. 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<sup>[42,68,69,71-76,78,79]</sup>. 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)<sup>[42,72,82]</sup>. 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<sup>[82]</sup>. 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<sup>[74,75,79]</sup>. Pharmacokinetic parameters, however, of gemcitabine, docetaxel, or paclitaxel/carboplatin, when co-administered with reovirus, were not appreciably different compared to receiving those agents alone<sup>[74,75,79]</sup>. 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<sup>[42,68,69,71-73,75,79]</sup>. When post-treatment viral shedding RT-PCR analyses were positive, they generally occurred within a few weeks (range 1-149 d) with some exceptions<sup>[42,68,69,71-73,75,79]</sup>. 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<sup>[76]</sup>. 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<sup>[76]</sup>.

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<sup>[42,71-73,75,76,78,80,81]</sup>. 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<sup>[72]</sup>. Similarly, in REO 013, replicating virus was recovered from lysates from surgical specimens in all 4 patients tested<sup>[76]</sup>. 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)<sup>[78]</sup>.

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<sup>[42,69]</sup>. Indeed, these observations have been somewhat

corroborated in a separate phase I trial (REO 005) that revealed increases in CD3<sup>+</sup>CD4<sup>+</sup> T-lymphocytes in 47.6% of patients, CD3<sup>+</sup>CD8<sup>+</sup> T-lymphocytes in 33% of patients, CD8<sup>+</sup> perforin/granzyme<sup>+</sup> T-lymphocytes in 23.8% of patients, CD3<sup>+</sup>CD56<sup>+</sup> 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<sup>[82]</sup>. Of note, there were no clear relationships between immune responses and reovirus dose, clinical response, or toxicity<sup>[82]</sup>.

### Phase II and III trials

An early multicenter, single-arm, open-label, phase II trial (REO 008) involved  $1 \times 10^{10}$  TCID<sub>50</sub> intratumoral injections of reovirus on days 2 and 4 with 4 Gy of local irradiation daily  $\times$  5 fractions (total 20 Gy per cycle) in the treatment of refractory or metastatic solid tumors<sup>[83]</sup>. Out of 14 evaluable patients, the best overall response was SD or better in 13 patients (93%)<sup>[83]</sup>. 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)<sup>[83]</sup>. 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<sup>[83]</sup>. The NCI-sponsored MAYO-MC0672 was a multicenter, single-arm, open-label, phase II trial pitting 60-min IV infusion of reovirus  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 every 28 d against metastatic melanomas<sup>[84]</sup>. 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)<sup>[84]</sup>. 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<sup>[84]</sup>. 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<sup>[84]</sup>. 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%)<sup>[84]</sup>. The most common treatment-related grade 3 or 4 AEs were fatigue (9.5%), hyponatremia (9.5%), and lymphopenia (9.5%)<sup>[84]</sup>.

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  $3 \times 10^{10}$  TCID<sub>50</sub> administered on days 1-5 every and given 28 d (personal communication). The best overall response was SD  $\geq$  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  $\geq$  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  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 with IV paclitaxel 175 mg/m<sup>2</sup> over 3 h and IV carboplatin AUC<sub>5</sub> over 30 min on day 1 every 21 d<sup>[85]</sup>. 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<sup>[85]</sup>.

REO 016 enrolled 37 patients with recurrent or metastatic NSCLC originally treated with IV reovirus in combination with IV paclitaxel 200 mg/m<sup>2</sup> and IV carboplatin AUC<sub>6</sub>, but due to grade 3 diarrhea and febrile neutropenia (1 each), the dosing regimen was amended to 60-min IV infusion of reovirus  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 with IV paclitaxel 175 mg/m<sup>2</sup> over 3 h and IV carboplatin AUC<sub>5</sub> (over 30 min) on day 1 every 21 d<sup>[86]</sup>. 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<sup>[86]</sup>. 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%)<sup>[86]</sup>. The most common AEs were fatigue, diarrhea, nausea, arthralgia/myalgia, and anorexia<sup>[86]</sup>. 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  $1 \times 10^{10}$  TCID<sub>50</sub> on days 1, 2, 8 and 9 with IV gemcitabine 800 mg/m<sup>2</sup> days 1 and 8 every 21 d<sup>[87]</sup>. 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<sup>[87]</sup>.

The open-label, single-arm phase II trial (REO 021) involved 60-min IV infusion of reovirus  $3 \times 10^{10}$  TCID<sub>50</sub> days 1-5 with IV paclitaxel 200 mg/m<sup>2</sup> over 3 h and IV carboplatin AUC<sub>6</sub> every 21 d in the treatment of recurrent or metastatic SCC of the lung<sup>[88]</sup>. 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%<sup>[88]</sup>. Of the 21 patients with > 6 mo follow-up, seven patients experienced PFS  $\geq$  6 mo (33.3%)<sup>[88]</sup>. The most common AEs were those expected of paclitaxel/carboplatin including neutropenia and thrombocytopenia and those expected of reovirus

such as fever and fatigue<sup>[88]</sup>. The only treatment-related serious AE was reversible grade 2 elevation in creatinine and blood urea nitrogen<sup>[88]</sup>.

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  $3 \times 10^{10}$  TCID<sub>50</sub> 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)<sup>[20]</sup>. Neutralizing antibodies similarly did not affect the efficacy of intratumoral reovirus in immune-competent rodent models of subcutaneous and intracranial glioblastoma<sup>[89]</sup>. 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<sup>[82]</sup>. 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 occurred by 3 wk of IV reovirus therapy which coincided with rising serum NARA titers<sup>[58]</sup>. 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<sup>[58]</sup>.

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<sup>[82]</sup>. 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<sup>[82]</sup>. 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<sup>[66]</sup>. 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<sup>[66,74,75,79]</sup>. All 3 combination regimens, however, have produced promising findings of clinical efficacy in various advanced malignancies and await further investigation in later trials<sup>[74,75,79]</sup>.

### **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<sup>[58]</sup>. 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<sup>[56]</sup>. Furthermore, reovirus has been associated with limb necrosis and death in approximately 50%-60% of reovirus-treated SCID mice<sup>[20]</sup>. 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<sup>[56]</sup>.

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<sup>2</sup> with at least 3 patients per cohort with a consistent dose of reovirus dose of  $3 \times 10^{10}$  TCID<sub>50</sub>/d<sup>[90]</sup>. The combination of cyclophosphamide and reovirus was well tolerated with few grade 3 toxicities including fever, diarrhea, neutropenia, and anemia<sup>[90]</sup>. However, cyclophosphamide did not have an effect at stimulating an antiviral response as NARA titers rose > 50 fold in all but one patient<sup>[90]</sup>. 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<sup>[90]</sup>.

### **Immune-mediated antitumor activity of reovirus**

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

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<sup>[95,96]</sup>. 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<sup>[92,96]</sup>. 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<sup>[46,92,97]</sup>. Importantly, further support has been offered in early clinical trials when phase I data showed increases in CD3<sup>+</sup>CD4<sup>+</sup> T-lymphocytes in 47.6% of patients, CD3<sup>+</sup>CD8<sup>+</sup> T-lymphocytes in 33% of patients, CD8<sup>+</sup>perforin/granzyme<sup>+</sup> T-lymphocytes in 23.8% of patients, CD3-CD56<sup>+</sup> NK cells in 28.6% of



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

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<sup>[92]</sup>. 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<sup>[98]</sup>. 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<sup>[99]</sup>. 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<sup>[99]</sup>. 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<sup>[100]</sup>.

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<sup>[86,87]</sup>. 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<sup>[101]</sup>. Checkpoint inhibition improved the ability of NK cells to kill reovirus-infected tumor cells and enhanced the CD8<sup>+</sup> 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)<sup>[68,69,71,72,78]</sup>. 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<sup>[84]</sup>. 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 monotherapy 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<sup>[2,4,6,7]</sup>.

The delivery of viruses to target tissues in sufficient numbers to produce a meaningful therapeutic effect has been a longstanding tenet of virotherapy<sup>[2]</sup>. 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<sup>[58]</sup>. 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

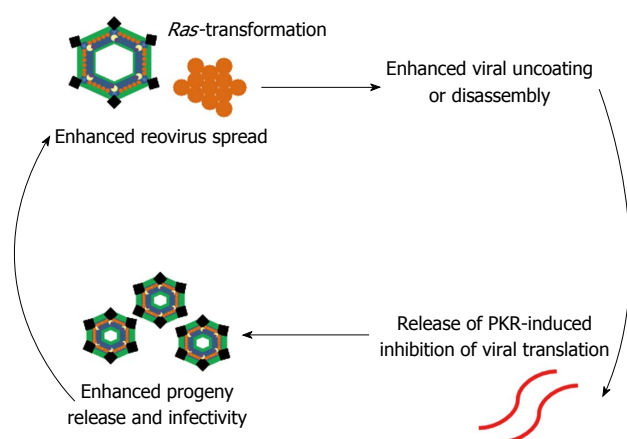
an attractive agent to use in combination with other therapies and, overall, makes combination clinical trials much more feasible. Furthermore, the mechanism of reovirus oncolysis offers synergistic potential when used with other agents due to differing pathways of inducing cancer cell death. These reasons 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<sup>[86,87]</sup>. 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<sup>[43,44]</sup>.

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<sup>[102]</sup>. 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- $\kappa$ B 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<sup>[102-105]</sup>. 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<sup>[106]</sup>. 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<sup>[99]</sup>. 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 disassembly. dsRNA-activated protein kinase (PKR), which in the presence of viral transcripts, normally phosphorylates eukaryotic initiation factor 2 $\alpha$  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<sup>[107-110]</sup>. 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<sup>[65]</sup>.

## 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<sup>®</sup>) 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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## 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



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**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<sup>[1]</sup>. The Centers for Disease Control and Prevention reports that 1/3 of the United States adult population, or 78.6 million, is obese<sup>[2]</sup>. The negative impact on the public health burden continues to increase with the number of overweight and obese individuals<sup>[3]</sup>. A quantitative meta-analysis of 33 United States studies lists the direct medical cost of obesity as \$1723 per-person<sup>[4]</sup>. 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<sup>[5]</sup>, type two diabetes mellitus<sup>[6]</sup>, and several different types of cancer<sup>[7]</sup>.

Clinical measures for obesity include having a body mass index (BMI) that is  $\geq 30$ ; clinical obesity can also be characterized by excessive accumulation of body fat<sup>[8]</sup>. BMI measures body fat using an individual's height to weight ratio<sup>[9]</sup>. Physicians, clinicians, nutritionists, and other scientists often assess an individual's BMI to determine obesity related health outcomes<sup>[10]</sup>. 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<sup>[11]</sup>. Clinicians and researchers are now correlating body fat and waist circumference to increased risks for cardiovascular disease, type-two diabetes, and cancer<sup>[12-14]</sup>. 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<sup>[11]</sup>.

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

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<sup>[18]</sup>. 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, endometrium, esophageal, kidney, liver, lung, ovarian, prostate, skin, and thyroid cancers<sup>[18]</sup>.

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<sup>[19]</sup>, 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)<sup>[18]</sup>. 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-RI or OB-Rb, with full signaling capabilities, carries out primary biological functions of leptin, while short OB-R isoforms induce secondary signaling pathways<sup>[18,20]</sup>.

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

JAK2 protein<sup>[18,22,23]</sup>. JAK2 phosphorylates three tyrosine residues (Tyr985, Tyr1077 and Tyr1138) on the intracellular portion of the OB-R<sup>[18,23,24]</sup>. 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<sup>[24]</sup>. However, these mechanisms become dysfunctional during obesity as leptin continue to rise until the body no longer responds to endogenous leptin signaling pathways<sup>[25]</sup>. 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<sup>[26]</sup>.

## 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<sup>[27]</sup>. 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<sup>[27]</sup>. Additionally, *ob/ob* and *db/db* mice show very low incidence of mammary tumors<sup>[27]</sup>. 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<sup>[26,28,29]</sup>. 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<sup>+</sup>) and unresponsive (ER<sup>-</sup>) breast cancer cells *in vitro*<sup>[30]</sup>. Moreover, inhibition of leptin signaling has been shown to abrogate ER<sup>+</sup> and ER<sup>-</sup> breast cancer growth<sup>[31]</sup>. 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<sup>[26,32,33]</sup>. 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*<sup>[30,34]</sup>. This was further assessed by Zheng *et al.*<sup>[35]</sup> who have confirmed that Cyclin D1 expression is regulated by active leptin signaling in the MMTV-Wnt-1 transgenic mouse<sup>[35]</sup>. 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<sup>[36]</sup>. These data suggest that leptin is essential in promoting S-phase cell cycle progression in breast cancer.

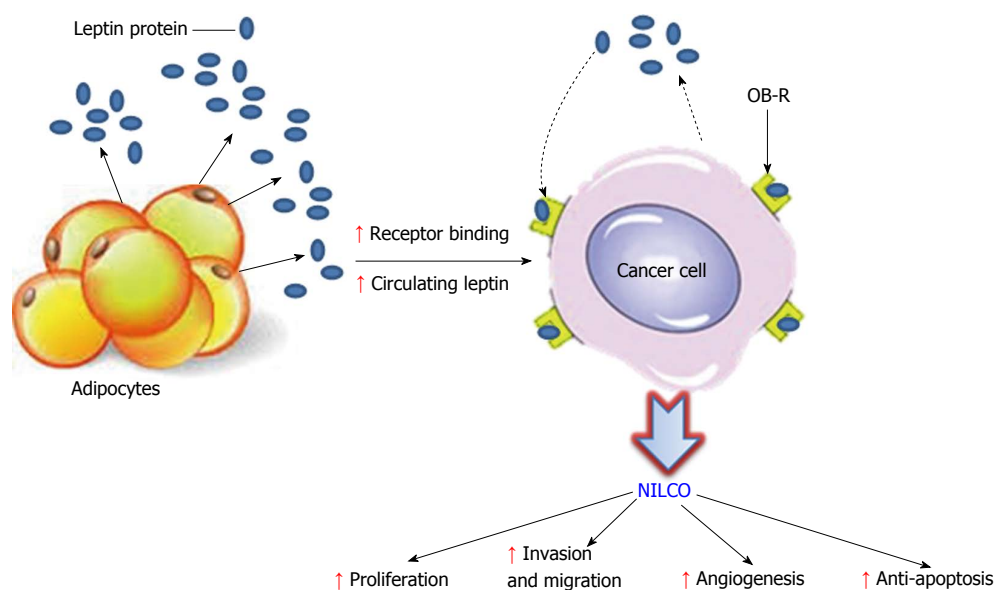
The effects of leptin signaling on proliferative pathways have also been linked to telomerase activity. Leptin has been shown to induce telomerase activity in MCF-7 breast cancer cells in a dose-dependent manner. Moreover, leptin signaling may be a key promoter of senescence evasion in human breast cancer cells *via* up regulation of telomerase activation<sup>[37]</sup>. 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.*<sup>[38]</sup>. These authors found an inverse relationship between the expression of apoptotic protein in TNBC treated with leptin<sup>[38]</sup>. Leptin induced Bcl-xL and Bcl-2 protein levels and increased survival of TNBC<sup>[38]</sup>. These survival mechanisms are punctuated by apoptosis evasion *via* leptin's ability to regulate Bcl-2 proteins in human breast cancer cells<sup>[38]</sup>.

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<sup>[39]</sup>. Additionally, VEGF/VEGFR-2 autocrine and paracrine actions in breast cancer have been shown to play an important role in cancer cell survival<sup>[28]</sup>.

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

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 intracytoplasmic



**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<sup>[29,71,85,89]</sup>. 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<sup>[42]</sup>. 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<sup>[43]</sup>.

Proliferation and acquisition of malignant features in breast cancer cells has illustrated the important role of leptin signaling and crosstalk between several cellular pathways<sup>[44]</sup>. 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<sup>[44]</sup> (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<sup>[44]</sup>.

Notch is a hallmark of breast cancer<sup>[44]</sup>. 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 intracytoplasmic tail *via* ADAM protease and  $\gamma$ -secretase, which have been shown to regulate cell differentiation<sup>[45]</sup>. Notably, our group has recently shown that leptin induces Notch activation in estrogen responsive and TNBC cells *in vitro* and *in vivo*<sup>[46]</sup>. Moreover, we have shown that leptin induced cell proliferation and migration are Notch dependent<sup>[46]</sup>. 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  $\gamma$ -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<sup>[44,46]</sup>.

NILCO biomarkers were found differentially expressed in ER<sup>+</sup>, ER<sup>-</sup> and TNBC tissues obtained from Chinese women. TNBC showed differential localization patterns of NILCO. TNBC showed fewer nuclei and cytoplasm positive for Notch4 and JAG1, but more cytoplasm 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  $\alpha$  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<sup>[32]</sup>.

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<sup>[47]</sup>.

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<sup>[18]</sup>.

### **Leptin, epithelial to mesenchymal transition and tumor stroma**

Leptin signaling induces adhesion, increases migration, and invasion in breast cancer cells *in vitro*<sup>[44]</sup>. Cancer cells that gain an increased ability to migrate and invade surrounding 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,  $\alpha$ v $\beta$ 3 integrin receptor and matrix metalloprotease 2, suggesting that leptin induces adhesion and migration processes<sup>[48]</sup>. Furthermore, leptin was shown to stimulate phosphorylation of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) *via* Akt activation that decreased GSK3 $\beta$ -LKB1-Axin complex formation and induced  $\beta$ -catenin, Wnt1 and MTA1 expression. Moreover, leptin-treated breast tumors hosted by mice showed increased expression of Wnt1, pGSK3 $\beta$ ,  $\beta$ -catenin and vimentin, but reduced E-cadherin expression. A novel crosstalk between leptin and MTA1/Wnt signaling was found in breast cancer cell-EMT<sup>[49]</sup>.

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<sup>[44,50]</sup>. 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<sup>[51]</sup>. The end result is an activation of pathways that confer stem-cell-like properties (OCT-4<sup>+</sup>/SOX2<sup>+</sup>) to the breast cancer cells<sup>[51]</sup>. 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<sup>[41,44]</sup>. 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<sup>[18,52,53]</sup>.

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<sup>[52]</sup>.

### **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<sup>+</sup> and/or progesterone responsive (PR<sup>+</sup>) breast cancers<sup>[54]</sup>. Additionally, TAM is indicated for reduction of contralateral BC risk<sup>[55]</sup>. Therefore, the National Cancer Institute recommends long-term TAM chemoprevention. To date more than 7 million patients a year use TAM<sup>[56]</sup>.

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<sup>[39,57]</sup>. 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<sup>[58]</sup>. In turn, leptin can transactivate ER, and increase aromatase activity, which leads to the induction of estrogen synthesis<sup>[59]</sup>. Reciprocally, estrogen signaling can induce leptin and OB-R expression, increase the development of vascular thrombosis<sup>[60]</sup> and impair TAM effects<sup>[58]</sup>.

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



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

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<sup>[63]</sup>. When treated *in vitro* with 2  $\mu\text{mol/L}$  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<sup>[63]</sup>. However, 72 h combination treatment of MCF-7 and MCF-7/HER2 cell lines with 2  $\mu\text{mol/L}$  TAM + 200 ng/mL leptin had a restorative effect on cell viability<sup>[63]</sup>. 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<sup>[63]</sup>. In contrast, MCF-7/HER2 cells treated with TAM alone had decreased expression of phosphorylated STAT3<sup>[63]</sup>. 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<sup>[63]</sup>. 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<sup>+</sup> 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<sup>[64]</sup>. 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<sup>[64]</sup>. 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<sup>[64]</sup>.

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<sup>+</sup> breast cancer patients who were treated with TAM<sup>[65]</sup>. Similarly, the Austrian Breast and Colorectal Cancer Study Group trial 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<sup>[66]</sup>. However, obese women who received TAM in combination with an aromatase inhibitor had worse outcomes than non-obese

counterparts receiving the same treatment<sup>[66]</sup>. These studies show that there may be strong associations between body weight and TAM efficacy. Azrad *et al.*<sup>[67]</sup> 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<sup>[67]</sup>.

Links between leptin levels and chemotherapeutic resistance mechanisms have also been identified in the MCF-7 breast cancer cell line *in vitro*<sup>[68]</sup>. MCF-7 cells were treated with 100 ng/mL of leptin for 10 d, on the 9<sup>th</sup> day cells were treated with 10  $\mu\text{mol/L}$  concentration of cisplatin (CIS)<sup>[68]</sup>. The effect of chronic leptin exposure on cell proliferation during CIS treatment was measured using Crystal Violet assays<sup>[68]</sup>. 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<sup>[68]</sup>.

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 chemoresistance in breast cancer. Increasing BMI of TNBC patients was associated with significantly more advanced disease and higher incidence and lower response rates to chemotherapeutics<sup>[69]</sup>.

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<sup>[70]</sup>. Importantly, leptin activates several molecules critically associated with BCSC, (*i.e.*, Notch, Akt, STAT3, nuclear factor- $\kappa\text{B}$ )<sup>[71-73]</sup>. 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<sup>[72]</sup>. We further tested the effects of leptin in human ER<sup>+</sup> (MCF-7) and TNBC cell lines (HCC1806) exposed to various concentrations of CIS<sup>[74]</sup>, sunitinib, paclitaxel, and doxorubicin<sup>[36]</sup>. 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)<sup>[36]</sup>. 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<sup>[75,76]</sup>. 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<sup>[77]</sup>.

EmCa can be classified as type I and type II<sup>[75]</sup>. 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<sup>[75]</sup>. Indeed, type I EmCa is less aggressive and less likely to spread to distant sites<sup>[75,78]</sup>. 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<sup>[75]</sup>. Moreover, type II EmCa is more likely to metastasize to distant sites<sup>[75,78]</sup>. Type II EmCa includes several subtypes (*i.e.*, papillary serous carcinoma and clear-cell carcinoma)<sup>[75]</sup>. Less frequent EmCa types, malignant mixed müllerian tumors or carcinosarcomas, are considered Type II tumors that represent approximately 4% of uterine cancers<sup>[78]</sup>.

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<sup>[77]</sup>. 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)<sup>[77]</sup>. 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<sup>[77]</sup>.

Obesity and EmCa incidence strongly correlate<sup>[7]</sup>. Approximately 40% of EmCa cases are related to obesity<sup>[75]</sup>. EmCa is more than three times as common in obese women when compared to normal healthy weight women<sup>[75]</sup>. 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<sup>[75]</sup>. 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<sup>[79]</sup>. 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<sup>[75]</sup>. In An3Ca, Ishikawa, and SK-UT2 (malignant endometrial epithelial) cell lines leptin regulates VEGF, IL-1 $\beta$ , LIF and their respective receptors. However, IL-1 $\beta$  was only increased by leptin in benign primary endometrial cells. IL-6, resistin, and tumor necrosis factor  $\alpha$  are additional factors involved in leptin oncogenic crosstalk in EmCa<sup>[18]</sup>. The short OB-R isoforms are expressed higher than the long OB-R isoform in EmCa<sup>[75,80]</sup>.

As previously described in this review, NILCO, a complex crosstalk between leptin and pro-angiogenic, inflammatory and mitogenic factors occurs in breast cancer<sup>[44]</sup>. Additionally, NILCO could also be present in EmCa<sup>[75]</sup>. 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<sup>[75]</sup>.

We have previously reported an interesting observation showing that NILCO components are differentially expressed in type I and type II EmCa<sup>[75]</sup>. 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<sup>[81]</sup>. However, estrogens are also synthesized by adipose tissue<sup>[82]</sup>. In fact, estradiol levels varied throughout the menstrual cycle between women with different body fat content<sup>[83]</sup>. 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<sup>[83]</sup>.

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<sup>[75]</sup>. Therefore, these molecules could alter female reproductive function and hormonal equilibrium especially after menopause in obese women<sup>[84]</sup>. 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<sup>[32]</sup>: 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<sup>[84]</sup>. Conversely, androstenedione and dehydroepiandrosterone showed no significant variations throughout the menstrual cycle<sup>[84]</sup>. Androstenedione levels were found to peak at ovulation<sup>[85]</sup>. 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<sup>[86]</sup>.

Also, the menstrual cycle may be influenced by the levels of serum leptin<sup>[87]</sup>. 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<sup>[87]</sup>. However, serum leptin levels were unchanged throughout the menstrual cycle of women with normal weight<sup>[87]</sup>.

## 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<sup>[88]</sup>. A positive significant association between waist circumference and PC was determined in a combined meta-analysis of cohort studies for the Asia-Pacific region<sup>[89]</sup>.

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			
	Control	Leptin	Leptin + CT	Leptin + CT + LI	Control	Leptin	Control	Leptin
Proliferation (%)	100	212 <sup>2</sup>	63 <sup>2</sup>	55 <sup>2</sup>	100	120 <sup>2</sup>	100	130 <sup>2</sup>
Survival <sup>1</sup> (%)	100	100					100	100
PCSC (%)	100	130-140			100	120-130		
Notch1	100	170 <sup>2</sup>			100	NC		
Notch2	100	147 <sup>2</sup>			100	75		
Notch3 (%)	100	NC			100	NC		
Notch4	100	71			100	146 <sup>2</sup>		
Tumorsphere formation (%)	100	197 <sup>2</sup>			100	184 <sup>2</sup>	100	221 <sup>2</sup>

<sup>1</sup>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); <sup>2</sup>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<sup>[90]</sup>. Overexpression of leptin was shown to significantly promote the growth of human PC xenografts and lymph node metastasis in mice<sup>[91]</sup>. 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<sup>[92,93]</sup>, 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)<sup>[94]</sup>. 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)<sup>[88]</sup>.

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<sup>+</sup>/CD44<sup>+</sup> markers in PC correlated to higher tumorsphere formation. Moreover, triple positive PC cells (CD24<sup>+</sup>/CD44<sup>+</sup>/ESA) were identified as PCSC<sup>[95]</sup>. Our preliminary data show that leptin significantly increased PCSC (CD24<sup>+</sup>/CD44<sup>+</sup>/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<sup>[96]</sup>. 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<sup>[97]</sup>. 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<sup>[98,99]</sup>.

It is believed that obesity may promote the progression of ER<sup>+</sup> breast cancer in post-menopausal obese women via increased production of the estrogens by adipocytes<sup>[100]</sup>. 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<sup>[101]</sup>. 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<sup>[102]</sup>. 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)<sup>[102]</sup>. Interestingly, 50% of the patients had circulating adiponectin levels that are the same as normal weight women<sup>[102]</sup>. 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<sup>[96,102,103]</sup>. 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<sup>[101-104]</sup>. 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<sup>[104]</sup>. 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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## 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 aetio-pathogenesis 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

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**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.

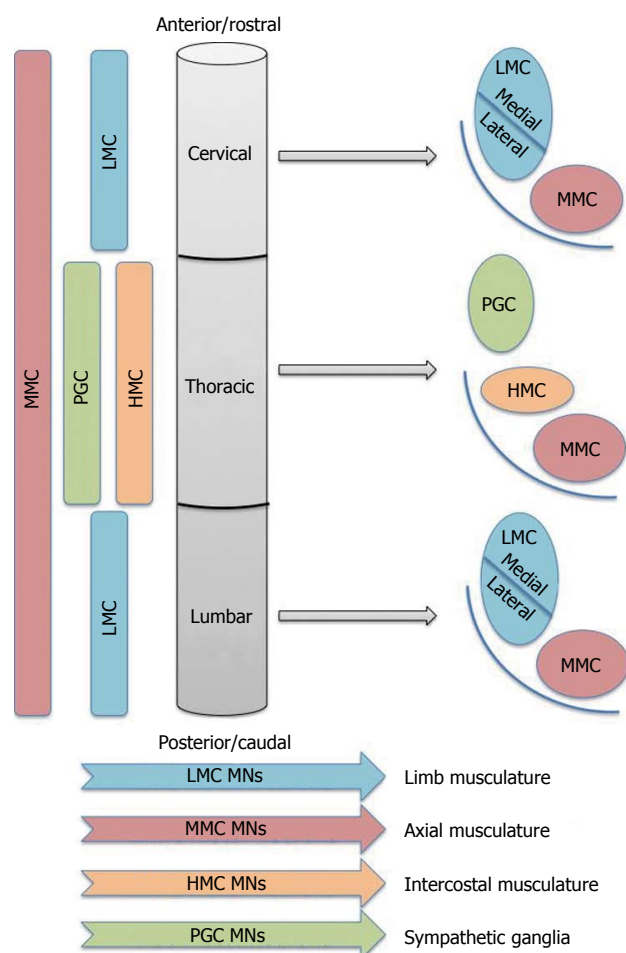
Lower MNs are anatomically positioned in the ventral horns of the spinal cord and motor nuclei within the brainstem; these in turn synapse at neuromuscular junctions and muscle spindles forming a final common pathway for voluntary movement. Spinal MNs are large, polarized cells with long axons, and are 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 dendritic 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<sup>[1]</sup>. 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<sup>[2,3]</sup>. 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<sup>[4-8]</sup>. 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<sup>[9-12]</sup>, invariably leading to fatal paralysis usually through respiratory failure<sup>[13,14]</sup>. The lifetime risk of MND is 1:400 in those of European ancestry<sup>[15]</sup>. 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<sup>[16-18]</sup>. 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. Sialorr-

hoea 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<sup>[19]</sup>. 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<sup>[20]</sup>. 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<sup>[21-23]</sup>. 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<sup>[24]</sup>. A study prior to the introduction of the Awaji criteria found that 29% of MND patients died without a diagnosis of definite MND<sup>[25]</sup>. The Awaji diagnostic criteria have been shown to increase the sensitivity of MND diagnosis<sup>[24,26]</sup>. 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<sup>[23]</sup>. 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<sup>[23]</sup>**

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	<i>e.g.</i> , 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 bulbar/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<sup>[27]</sup>.

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<sup>[28]</sup>. Early cortical hyperexcitability, which may reflect glutamate excitotoxicity, precedes lower MN involvement in MND, and through the course of the disease this hyperexcitability decreases<sup>[28-32]</sup>. 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<sup>[33]</sup>. Three hypotheses for MN death have been proposed: (1) a “dying-forward” phenomenon, where disease 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<sup>[34]</sup>.

Various staging systems have been devised to measure disease progression in MND<sup>[35-40]</sup>. Individuals can progress through the disease at very variable rates<sup>[41,42]</sup>, 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<sup>[35,36]</sup>. Furthermore, incremental stages correspond to decreasing function and health utility, and can be used in cost-benefit analyses of new treatments<sup>[38]</sup>. 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<sup>[43]</sup>. 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<sup>[43,44]</sup>.

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<sup>[45-48]</sup>. 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<sup>[19,49]</sup>. 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<sup>[50,51]</sup>.

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<sup>[52]</sup>. 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)<sup>[53]</sup>, a highly conserved, ubiquitously expressed and multifunctional nuclear protein with

both DNA and RNA binding capacities<sup>[54-56]</sup>. 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)<sup>[43,57]</sup>.

Other recent discoveries identified MND-causing gene mutations in TDP-43 and FUS<sup>[58,59]</sup>; 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<sup>[60,61]</sup>, 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<sup>[43,58,59,62-66]</sup>.

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<sup>[67-69]</sup>. Non cell-autonomous injury has also recently been implicated in sporadic MND, raising the possibility of common pathogenic mechanisms<sup>[70,71]</sup>.

The discovery of induced pluripotent stem cells (iPSC) enables patient-specific fibroblasts to be virally transduced with up to 4 transcription factors and "re-programmed" into embryonic-like stem cells<sup>[72]</sup>. 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<sup>[73-76]</sup>. 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<sup>[71,77-79]</sup>. 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<sup>[80]</sup>. Several studies have also confirmed the utility in this model system as a pre-clinical test-bed for drug discovery<sup>[81-83]</sup>, including the practical feasibility of high throughput automated approaches<sup>[84]</sup>.

## 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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## 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 conducted 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 padoporfin 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

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**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<sup>[1,2]</sup>.

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<sup>[3]</sup>. The currently available treatment modalities for localised PCa (LPCa) are radical therapy (RT) and active surveillance. RT consists in ablation of entire prostate gland<sup>[3-6]</sup> while active surveillance includes monitoring of serum PSA concentration and repeat prostate biopsies to select patients for curative therapy<sup>[6-8]</sup>. 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<sup>[4,9-11]</sup>, whereas active surveillance presents a considerable risk of cancer progression, metastases, and patients mortality<sup>[6,12-14]</sup>. 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<sup>[15]</sup>. Moreover, frequent medical exams during active surveillance may create adverse effects and deteriorate patient quality of life<sup>[6]</sup>. 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<sup>[16]</sup>.

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<sup>[17-20]</sup>. 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<sup>[3,21-24]</sup>, although there are no maximum tumour volume over which focal therapy is recommended<sup>[19,25]</sup>. 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)<sup>[19,20]</sup>.

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)<sup>[24,26,27]</sup>.

## 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<sup>[28-30]</sup>.

The development of prostate PDT in particular has accelerated rapidly in past few decades<sup>[31-33]</sup>. 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<sup>[34]</sup>, meso-tetra-(m-hydroxyphenyl)chlorin<sup>[35,36]</sup>, 5-aminolevulinic acid<sup>[37]</sup>, motexafin lutetium (Lu-Tex)<sup>[38-40]</sup> or temoporfin<sup>[41]</sup>, 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<sup>[42,43]</sup>. 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<sup>[27,44]</sup>. 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<sup>[35,45]</sup>. Finally, this strategy does not preserve adjacent structures of prostate gland, such as NVB, rectum or urinary sphincter<sup>[27,45,46]</sup>.

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<sup>[47-50]</sup>. 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<sup>[47,51,52]</sup>. 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<sup>[53]</sup>. 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<sup>[54,55]</sup>.

## PADOPORPHIN AND PADELIPORPHIN - PHOTSENSITIZERS USED IN FOCAL VTP OF LPCa

Padoporphin (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<sup>[55]</sup>. Preparing of water-soluble formulations of padoporphin 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<sup>[56,57]</sup>. Pharmacokinetic study using murine models showed that padoporphin in Cremophor® formulation, after bolus administration to healthy mice at a dose of 6 mg/kg<sup>[47]</sup> or to EMT-6 breast cancer-xenografted mice at a dose of 5 mg/kg<sup>[58]</sup>, 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)<sup>[47,58,59]</sup>. The total rate of clearance and the apparent volume distribution of padoporphin in the tumour bearing mice are estimated to be 11 mL/h and 0.9 mL respectively<sup>[58]</sup> while the maximal plasma concentration ( $C_{max}$ ) is 19 mg/L, at the maximal plasma concentration time ( $t_{max}$ ) of 5 min<sup>[58]</sup>. Similarly, in canine models, the maximum concentration of padoporphin in circulation after intravenous injection occurred in less than 10 min<sup>[60]</sup>. Padoporphin 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%)<sup>[61]</sup>.

In contrast, padeliporphin (palladium bacteriopheophorbide monolysine taurine; WST-11; Tookad® Soluble; Stakel®) is soluble in aqueous solutions; its logP is -0.19<sup>[55,62]</sup>. 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<sup>[55]</sup>. The  $C_{max}$  of padeliporphin 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<sup>[47]</sup>. In order, after 20 min intravenous infusion at a dose of 10 mg/kg into healthy rats, the half-life of padeliporphin was 7.5 min<sup>[47]</sup>. Padeliporphin is characterised by minimal extravasation from vasculature and therefore remains in circulation even at high doses<sup>[47,63]</sup>. Contrary to padoporphin, padeliporphin binds primarily to HDP (about 80%) and poorly to LDL and HDL (5% and 15%,

respectively)<sup>[61]</sup>. Preclinical studies in animal models showed that padeliporphin-mediated photosensitisation causes occlusion of the full tumour vasculature in a few minutes of treatment<sup>[50,54,55,64]</sup>. Thus, padeliporphin appears to have a higher therapeutic index and to be far easier to use in the VTP treatment of prostate cancer, comparing to padoporphin<sup>[27]</sup>.

As padoporphin and padeliporphin in hydrophilic media present absorption maxima in the IR-A region (700-2500 nm)<sup>[55,65]</sup>, 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<sup>[66,67]</sup>. The advantage of this radiation is its considerably deeper penetration into human prostate tissues (0.57 cm for  $\lambda = 763$  nm) comparing to red light (about 10 mm for  $\lambda = 633$  nm), usually used in conventional PDT<sup>[68,69]</sup>.

In the clinical conditions, photosensitizers are usually delivered in form of infusion injected using a syringe pump through intravenous delivery line<sup>[70]</sup>. For delivery of padoporphin, which is not soluble in water, Weersink *et al.*<sup>[70]</sup> 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<sup>[70]</sup>.

The short  $t_{max}$  of padoporphin and padeliporphin implies short DLI value (10 min for both these dyes), as was shown in clinical studies<sup>[44,56,57,66,71,72]</sup>. This suggests that administration of drug and radiation may be conducted in one clinical visit<sup>[44]</sup>. 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<sup>[44,66]</sup>.

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<sup>[47]</sup>. For serum monitoring of these photosensitizing dyes, inductively coupled plasma mass spectrometry<sup>[47]</sup>, graphite furnace atomic absorption at a wavelength 247.6 nm (palladium atomic absorption line)<sup>[58]</sup> or *in situ* absorption spectroscopy after serum protein ultracentrifugation<sup>[61]</sup> may be used, however, in clinical practice, the effect and not concentration of these drugs are usually monitored during VTP<sup>[62]</sup>.

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

<sup>1</sup>Results obtained for mice with transplanted breast tumor cells after 5 mg/kg intravenous injection; <sup>2</sup>Results obtained for healthy mice after 6 mg/kg intravenous injection; <sup>3</sup>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<sup>[70]</sup>. 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<sup>[70,73]</sup>.

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<sup>[70]</sup>. 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<sup>2</sup>). 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<sup>[67,74]</sup>.

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<sup>[75]</sup>.

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<sup>[5,70]</sup>. Johansson *et al*<sup>[76]</sup> 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<sup>[76]</sup>. 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<sup>[76]</sup>. As shown by Xu *et al*<sup>[77]</sup>, 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<sup>[78]</sup>. Irradiation can be applied simultaneously with photosensitizer administration, after ending of this administration or at the point of maximal serum concentration of photosensitizer<sup>[60]</sup>. For delivery of conformal radiation, the use of functional optical fibres may be necessary. Rendon *et al*<sup>[79,80]</sup> 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<sub>2</sub>) 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<sub>2</sub> are documented in the parenchymal cells of malignant prostate gland<sup>[46,81]</sup>. 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<sup>[51,70]</sup>. This imaging method represents spatial and temporal changes in oxygenation, flow and volume of blood<sup>[82]</sup>.

## CLINICAL TRIALS

### Phase I trials

Weersink *et al*<sup>[83]</sup> 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<sup>2</sup>. 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<sup>2</sup>, 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<sup>[83]</sup>.

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*<sup>[56]</sup>. 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<sup>2</sup>, 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<sup>[56]</sup>.

### Phase I / II trials

Gertner *et al*<sup>[84]</sup> 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<sup>[85]</sup>. 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<sup>[84]</sup>.

Arumainayagam *et al*<sup>[86]</sup> 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  $\leq 7$  and PSA < 20  $\mu\text{g/L}$  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<sup>®</sup> for intravenous administration. For this reason, the clinical trial has been withdrawn<sup>[86]</sup>.

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<sup>[87]</sup>.

Azzouzi *et al*<sup>[88]</sup> 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 1<sup>st</sup>, 3<sup>th</sup>, and 6<sup>th</sup> 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 6<sup>th</sup> 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 6<sup>th</sup> 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<sup>[62]</sup>.

### Phase II trials

Trachtenberg *et al*<sup>[57]</sup> 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 ( $\lambda = 763$  nm), established in computer-assisted treatment plan. A complete response required radiation fluences of at least 23 J/cm<sup>2</sup> 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<sup>[57]</sup>.

Arumainayagam *et al*<sup>[72]</sup> 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<sup>[72]</sup>. 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<sup>[88]</sup>.

Quoraishi *et al*<sup>[74]</sup> 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 treatment. 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<sup>[74]</sup>.

Eymerit-Morin *et al*<sup>[89]</sup> 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<sup>[89]</sup>.

Steba Biotech<sup>[90]</sup> and Azzouzi *et al*<sup>[91]</sup> 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, padeliporfin 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<sup>[91]</sup>. 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<sup>[90]</sup>.

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<sup>[92]</sup>. 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*<sup>[67]</sup>. 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<sup>[20,67,92]</sup>.

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 padoporphin-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<sup>[57,67]</sup>.

### 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 padoporphin-mediated VTP treatment of prostatic carcinoma. According to treatment protocol padoporphin 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<sup>[93]</sup>.

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  $\pm 5$  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<sup>3</sup> 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 <i>et al</i> <sup>[83]</sup>
I	24	Padoporfin, 0.1-2 mg/kg	763 nm, 100, 230 and 360 J/cm	Trachtenberg <i>et al</i> <sup>[56]</sup>
I / II	15	Padoporfin, 0.1-2 mg/kg	763 nm, 100 J/cm	Gertner <i>et al</i> <sup>[84]</sup>
I / II	34	Padoporfin, 2 mg/kg	763 nm, 100-300 J/cm	Arumainayagam <i>et al</i> <sup>[86]</sup>
I / II	30	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	<a href="https://clinicaltrials.gov/ct2/show/NCT00946881">https://clinicaltrials.gov/ct2/show/NCT00946881</a> <sup>[87]</sup>
II	28	Padoporfin, 2 mg/kg	763 nm, 0.1-1000 J/cm	Trachtenberg <i>et al</i> <sup>[57]</sup>
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 J/cm	Arumainayagam <i>et al</i> <sup>[72]</sup>
II	40	Padeliporfin, 2-6 mg/kg	753 nm, 200 J/cm	Quoraishi <i>et al</i> <sup>[74]</sup>
II	85	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi <i>et al</i> <sup>[88]</sup>
II	56	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Eymerit-Morin <i>et al</i> <sup>[89]</sup>
II	86	Padeliporfin, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	<a href="https://clinicaltrials.gov/ct2/show/-NCT00975429">https://clinicaltrials.gov/ct2/show/-NCT00975429</a> <sup>[90]</sup>
II	117	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi <i>et al</i> <sup>[62]</sup>
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 J/cm	Moore <i>et al</i> <sup>[67]</sup>
II	40	Padeliporfin, 2, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	<a href="https://www.clinicaltrials.gov/ct2/show/NCT00707356">https://www.clinicaltrials.gov/ct2/show/NCT00707356</a> <sup>[92]</sup>
II / III	86	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi <i>et al</i> <sup>[91]</sup>
II / III	16	Padoporfin, 2 mg/kg	763 nm, no information on radiation fluence	<a href="https://www.clinicaltrials.gov/ct2/show/-NCT00312442">https://www.clinicaltrials.gov/ct2/show/-NCT00312442</a> <sup>[93]</sup>
II / III	1	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	Azzouzi <i>et al</i> <sup>[94]</sup>
II / III	19	Padeliporfin, 4 and 6 mg/kg	753 nm, 200 and 300 J/cm	Lebdai <i>et al</i> <sup>[95]</sup>
III	81	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	<a href="https://clinicaltrials.gov/ct2/show/-NCT01875393">https://clinicaltrials.gov/ct2/show/-NCT01875393</a> <sup>[96]</sup>
III	400	Padeliporfin, 4 mg/kg	753 nm, 200 J/cm	<a href="https://clinicaltrials.gov/show/-NCT01310894">https://clinicaltrials.gov/show/-NCT01310894</a> <sup>[97]</sup>

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 padeliporfin. 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<sup>[68,88,94]</sup>.

Lebdai *et al*<sup>[95]</sup> 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<sup>[95]</sup>.

### 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<sup>[96]</sup>.

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 padeliporfin. The procedure will be the same as in the trial described above. This trial is not yet enrolling participants<sup>[97]</sup>.

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 conducted 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<sup>[5,70]</sup>.

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<sup>[44,97]</sup>. Multiparametric MRI with gadolinium contrast may be suitable for detection and characterisation of therapy progress during VTP treatment<sup>[98-101]</sup>. 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<sup>[102,103]</sup>. In order, rules-based approach would involve specific fibre density, or specific limits at different prostate boundaries<sup>[77]</sup>. As some authors indicate the low costs of photochemotherapeutic methods as their advantage over other strategies of prostate cancer therapies<sup>[17]</sup>, a systematic cost-effectiveness analysis for VTP application in PCa treatment is indispensable.

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## 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

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**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<sup>[1]</sup>. Smoking and alcoholism are two well known predisposing factors<sup>[2]</sup>. Head and neck squamous cell carcinoma (HNSCC) is reported to be the sixth common cause of cancer mortality throughout the world<sup>[2]</sup>.

There is no significant improvement in the mortality rates even with continuous research and trials in the field of diagnostics and therapeutics<sup>[3]</sup>. As compared to other cancers like breast, cervix and colorectal, the five-year survival rate of HNSCC after diagnosis is significantly lower<sup>[4,5]</sup>. The reason for this could be failure in early diagnosis and insufficient effectiveness of therapeutic modalities<sup>[6,7]</sup>. The predominant cause of mortality in HNSCC is regional and/or distant metastatic spreading of tumor cells from primary site<sup>[8]</sup>. 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<sup>[9,10]</sup>. The major reason put forward for this trend include a lack of appropriate screening biomarkers<sup>[11]</sup>. 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<sup>[12]</sup>. 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<sup>[13]</sup>.

### CXCR4

The importance of CXCR4 in tumour progression and organ-specific metastasis in patients with HNSCC has been reported by a number of authors<sup>[14,15]</sup>. 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<sup>[16]</sup>. 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<sup>[17]</sup>. 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<sup>[17]</sup>. 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<sup>[18]</sup>. 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<sup>[19]</sup>. 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<sup>[20]</sup>. There are reports in literature suggesting that HNSCC with HPV has a favorable prognosis and that, it in fact, is a distinct clinicopathological entity<sup>[21]</sup>. 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<sup>[22]</sup>. Thus, HPV DNA may act as a diagnostic and prognostic marker in patients of HNSCC.

It is of interest to know that adding p16<sup>INK4A</sup> 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<sup>INK4A</sup>(+) types of tumors have better prognosis<sup>[23]</sup>. As reported by Danish Head and Neck Cancer Group 5 trial, p16<sup>INK4A</sup>(+) tumors appeared to be associated more strongly with poor histopathologic differentiation as compared to the p16<sup>INK4A</sup>(-) ones, but the difference was not statistically significant, indicating that p16<sup>INK4A</sup> alone is not an adequate marker<sup>[24]</sup>. In the study of panitumumab efficacy in patients with recurrent and/or metastatic head and neck cancer (SPECTRUM), the authors reported that the p16<sup>INK4A</sup> status of the tumor might have significant bearings in designing future trials in cases of recurrent or metastatic HNSCC<sup>[25]</sup>.

## 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<sup>[26]</sup>. MSI analysis in tumor cell DNA is of value in detection of pre-malignant conditions like erythroplakia and leukoplakia<sup>[1]</sup>. 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<sup>[27]</sup>. 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<sup>[27,28]</sup>. 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%<sup>[29-31]</sup>. 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<sup>[31]</sup>.

MSI analyses have the disadvantage of lack of uniformity in selection of different methods or the type and number of markers evaluated<sup>[32]</sup>. 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<sup>[30]</sup>. A specificity of 96% in salivary specimens for methylation specific polymerase chain reaction has been reported for detection of HNSCC<sup>[33]</sup>. Whereas it was observed to be 90% in salivary samples and 72% for serum samples in yet another study<sup>[34]</sup>. The lower rate of promoter hypermethylation may be due to dilution with normal, non-methylated DNA from normal mucosal areas<sup>[34]</sup>. 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<sup>[35,36]</sup>.

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<sup>[37-39]</sup>. Besides migration of tumor cells, metalloproteinases (MMPs) play a significant role in providing a micro-environment conducive for the growth and angiogenesis of tumors. These also help in cellular differentiation, proliferation and apoptosis in tumor tissues<sup>[38]</sup>. 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<sup>[40,41]</sup>. 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<sup>[42-45]</sup>.

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

## 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<sup>[12]</sup>. 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)<sup>[50,51]</sup>. 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<sup>[52,53]</sup>.

Zimmermann *et al.*<sup>[54]</sup> reported that four mRNAs (OAZ, SAT, IL-8 and IL-1 $\beta$ ) 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<sup>[55]</sup>. 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<sup>[56]</sup>. miRNAs, by virtue of their vast range of consequences may act both as oncogenes and tumor suppressor genes<sup>[57]</sup>. 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<sup>[58]</sup>. 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<sup>[59]</sup>.

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<sup>[60]</sup>. Park *et al*<sup>[61]</sup> 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<sup>[62,63]</sup>. Dereglulation 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<sup>[64]</sup>. 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<sup>[65]</sup>.

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<sup>[64]</sup>. 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<sup>[66]</sup>. 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<sup>[67-69]</sup>. 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<sup>[70-73]</sup>. 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<sup>[74]</sup>. Expression of MAGE has also been shown in the sputum samples of patients with HNSCC<sup>[75]</sup>. 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<sup>[76]</sup>. Other studies have also reported 85.5%-90% expression rate of MAGE in HNSCC tissue<sup>[77,78]</sup>. It may help in initiating target specific immunotherapy in these patients<sup>[79]</sup>. According to a recent report by Lee *et al*<sup>[80]</sup> 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<sup>[81]</sup>.

## 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<sup>[82]</sup>. 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<sup>[83]</sup>. Increased frequency of centrosomal abnormalities has also been seen in OSCC in cells with spindle checkpoint protein CDC20 overexpression<sup>[84]</sup>. 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 over-expressed.

## ACTIN AND MYOSIN

These are cytoskeletal proteins responsible for cell motility and invasion which are important components of epithelial tumorigenesis<sup>[85]</sup>. 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<sup>[86]</sup>. Increased actin isoforms have been observed in invasive basal cell carcinoma<sup>[87]</sup>, squamous cell carcinoma of cervix<sup>[88]</sup> and esophagus<sup>[89]</sup> and invasive OSCC<sup>[90]</sup>. Increase in myosin abundance has been observed in proteomics of tissue from OSCC region<sup>[91]</sup>. However, Turhani

*et al.*<sup>[92]</sup> 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<sup>[93]</sup>. CKs are expressed in a number of combinations depending on the type of epithelial cell of origin<sup>[94]</sup>. They are further divided into two subtypes (I and II) that are generally coexpressed<sup>[95,96]</sup>. These are found to be overexpressed in OSCC tissue as compared to normal mucosa<sup>[93]</sup>. Overexpression of cytokeratins has been related with tumor progression and prognosis<sup>[97]</sup>. Constitutive expression of cytokeratin-17 (CK-17) in the lungs is only found in the normal basal cells<sup>[98]</sup>. It is now emerging as a tissue-specific immunohistochemical biomarker in squamous cell carcinoma of larynx<sup>[99]</sup>. CK-17 mRNA overexpression has been demonstrated in OSCC by few authors<sup>[96,99,100]</sup>. 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 syncytial virus infected epithelial cells also<sup>[95]</sup>.

As CK-6 and CK-16 are found to be constitutively expressed in mucosal stratified squamous epithelia, they may be regarded 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<sup>[101]</sup>.

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<sup>[102]</sup>. 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<sup>[103]</sup>. 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<sup>[102,104]</sup>. p53 is associated with maintenance of cellular integrity and is regarded as guardian of genome<sup>[105]</sup>. 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<sup>[103,105-108]</sup>. 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<sup>[109,110]</sup>. Survival rate has also

been reported to be higher in p53 negative patients as compared to those who are positive for p53 mutations<sup>[102]</sup>. 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<sup>[111]</sup>.

## EUKARYOTIC TRANSLATION FACTOR 4E

It is a protein involved in the initiation of protein synthesis<sup>[112]</sup>. 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<sup>[113,114]</sup>. Overexpression of this protein in mice has been found to be associated with a number of malignancies like lymphomas, angiosarcomas, hepatomas and carcinoma lung<sup>[115]</sup>. An expression of 100% in HNSCC has been reported in some studies<sup>[116,117]</sup>. 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<sup>[113,118-121]</sup>. Another study has reported overexpression of eIF4E in tumor free surgical margins to be related to loco-regional recurrence in patients of HNSCC<sup>[122]</sup>.

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<sup>[123]</sup>. The dominant moderator of mismatch repair in HNSCC is promoter hypermethylation rather than direct mutation<sup>[124]</sup>. 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<sup>[124]</sup>. Multiple (5-7) risk NER genotypes have been associated with a 2.4-fold increased relative risk of second primary HNSCC<sup>[124]</sup>. The inactivation of these DNA repair genes may be linked to carcinogenesis by decreasing genomic stability and producing certain genetic alterations<sup>[125]</sup> (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 MAGE	Role in regulation of gene expression Suppresses apoptosis	Tumor tissue/saliva Biopsy specimen/saliva	Early detection Prognosis and in selecting targeted immunotherapy	Clinical validation required 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 transformation 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.  $^{18}\text{F}$ -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<sup>[126,127]</sup>. 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<sup>[128]</sup>. 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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## 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

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**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)<sup>[1]</sup>.

This term, PEComa, introduced by Zamboni *et al.*<sup>[2]</sup>, includes a group of tumors with distinctive perivascular epithelioid cells such as angiomylipomas, lymphangio-myomatosis, clear cell sugar tumor of the lung and the so-called PEComa, that have been described in various organs and tissues, including the skin<sup>[3-13]</sup>. In any case, PEComa are exceedingly rare; have been described in the pancreas<sup>[2]</sup>, pelvic cavity<sup>[14]</sup>, uterus<sup>[15]</sup>, prostate<sup>[16]</sup>, urinary bladder<sup>[17]</sup>, digestive tract<sup>[18]</sup>, vulva<sup>[18]</sup>, heart<sup>[18]</sup>, trachea<sup>[19]</sup>, lymph node<sup>[7]</sup>, breast<sup>[20]</sup>, bone<sup>[21]</sup> and soft tissues<sup>[22]</sup>. 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"<sup>[13]</sup>.

### "PEComa"

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

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<sup>[1]</sup>.

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<sup>[23]</sup>.

## 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

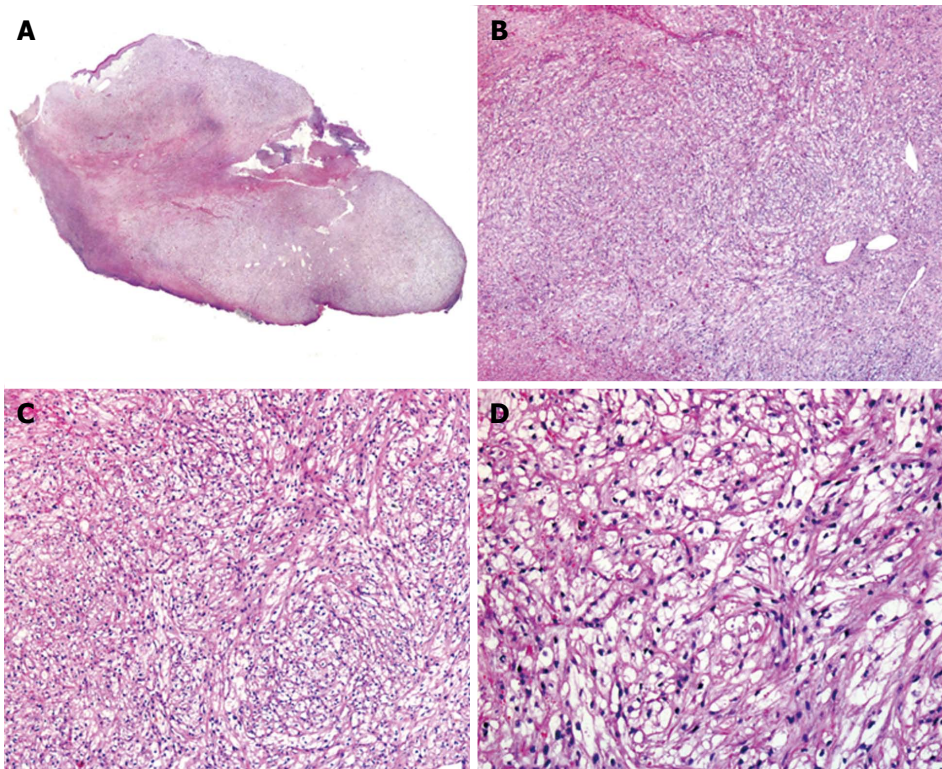
the back. They usually behave in a benign fashion<sup>[8]</sup>, although malignant examples have also been reported<sup>[7]</sup>. They typically appear in middle-aged adult females<sup>[24]</sup>. In our review of literature, we have found described 34 "legitimate" primary cutaneous PEComa<sup>[23]</sup>. Some of these neoplasms may be associated with tuberous sclerosis complex<sup>[4]</sup> but cutaneous lesions are mostly solitary lesions with no other associated anomalies<sup>[24]</sup>.

## 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)<sup>[13]</sup>. 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<sup>[7]</sup>. Up to 15% of PEComa present cords of neoplastic cells in a desmoplastic stroma<sup>[25]</sup>. 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<sup>[4]</sup>. 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) HMB45 [human melanoma black 45, the most sensitive (expressed in 100% of reported PEComa<sup>[8]</sup>)]; (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<sup>[24]</sup>); and smooth muscle actin (SMA), that may be the most sensitive marker within this group<sup>[4,26-28]</sup>. It is important to underline that up to 30% of visceral PEComa stain positive with S100 protein<sup>[4]</sup>.

Pusiol *et al.*<sup>[29]</sup> 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<sup>[29]</sup>.

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<sup>[30]</sup>. 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<sup>[4]</sup>

Features	Definition
Tumor size greater than 5 cm	Benign (none criteria)
Infiltrative growth pattern	
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 premelanosomes<sup>[12,26,27]</sup>.

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<sup>[7,31]</sup>; one of them a scalp lesion that lately metastasized to a regional lymph node<sup>[7]</sup>.

Criteria for diagnosis of "malignant PEComa" have been proposed by Folpe *et al*<sup>[4]</sup> (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<sup>[12]</sup>. 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<sup>[5]</sup>, as well as alterations on chromosome 16p (TSC2); this has been previously reported in angiomyolipomas<sup>[5]</sup> 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<sup>[32]</sup>. In visceral PEComa these alterations produce a constitutive activation of the mTORC1 pathway<sup>[33]</sup>. Some soft tissue PEComa in patients without tuberous sclerosis complex are immunohistochemically positive for TFE3<sup>[34,35]</sup>, 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<sup>[36]</sup>.

Finally, a recent study of Charli-Joseph *et al*<sup>[23]</sup> 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 4EBP1 as the ideal immunohistochemical panel for the evaluation of these

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

## DIFFERENTIAL DIAGNOSIS

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

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<sup>[41]</sup>.

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<sup>[42,43]</sup>. 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<sup>[44]</sup>.

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<sup>[45]</sup>.

Primary extracranial 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<sup>[46,47]</sup>.

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<sup>[48]</sup>. 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<sup>[49]</sup>. Even when SMA may be positive in desmoplastic melanoma<sup>[50,51]</sup>, 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<sup>[52]</sup>.

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<sup>[33]</sup>.

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<sup>[53]</sup>. Some authors consider that this tumor is possibly associated with PEComa, but still remains considered as a different entity based on the negativity for melanocytic markers<sup>[54]</sup>. Finally, Tomasini *et al.*<sup>[55]</sup> 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 heman-giopericytoma-like features<sup>[55]</sup>; this tumor was positive for h-caldesmon, SMA, CD13, CD68 and NKIC3<sup>[55]</sup>.

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<sup>[56]</sup>.

## TREATMENT

As most PEComa are benign tumors, surgical removal is curative<sup>[1]</sup>.

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<sup>[57]</sup> were in this location.

Besides surgery, drugs inhibiting the activation of mTOR, such as rapamycin, may be useful<sup>[58-62]</sup>. As patients with tuberous sclerosis have abnormalities in the *TSC2* gene and that activates mTOR leading tumorigenesis, 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<sup>[63]</sup>.

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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; Amino-transferase "common" reference intervals; Creatinine enzymatic method

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**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<sup>[1-4]</sup>. 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<sup>[5,6]</sup>. The International Organization for Standardization (ISO) standard 15189 require that "biological reference intervals shall be periodically reviewed"<sup>[7]</sup>, 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<sup>[8]</sup>.

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<sup>[9]</sup> 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<sup>[10-15]</sup>.

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<sup>[16,17]</sup>. 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 outgrew 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 assessment programme for medical biochemistry laboratories is formed modularly 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<sup>[18-21]</sup> 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<sup>[22]</sup>. 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<sup>[22]</sup>. To achieve this result it is necessary to harmonize the entire



**Table 1** Reference intervals for creatinine concentrations<sup>[31]</sup>

Age (gender) group	Percentile value, $\mu\text{mol/L}$	
	2.5 <sup>th</sup>	97.5 <sup>th</sup>
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-postanalytical 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 coordinate 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)]<sup>[22,23]</sup>. 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<sup>[24]</sup>, it was shown that the application of metrological traceability has a great practical potential and global value. This was confirmed through<sup>[24]</sup>: 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 commutable 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<sup>[23,25]</sup>.

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<sup>[26,27]</sup>. 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 commutable clinical samples required to maintain the process of harmonization<sup>[26,27]</sup>. 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](http://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<sup>[27]</sup> 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<sup>[28-30]</sup>. Serum creatinine concentrations were measured using specific enzymatic method traceable to the IDMS method in comparison to the uncompensated Jaffe kinetic creatinine method<sup>[31]</sup>. 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<sup>[32]</sup>.

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<sup>[33-35]</sup>. According to the CLSI approved guideline, validation of reference intervals is advisable<sup>[36]</sup>.

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<sup>[37-39]</sup>.

## 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<sup>[36]</sup>.

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%) were 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<sup>[10,14,15,40-44]</sup>.

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<sup>[45]</sup>.

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<sup>[46]</sup>. 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 °C, TRIS buffer, L-alanine, $\alpha$ -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	$\geq 20$	12-48	[14]	
			Female	$\geq 20$	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 °C, TRIS buffer, L-aspartate, $\alpha$ -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	$\geq 20$	11-38	[14]	
			Female	$\geq 20$	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

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**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<sup>[1-3]</sup>. 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<sup>[4]</sup>.

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

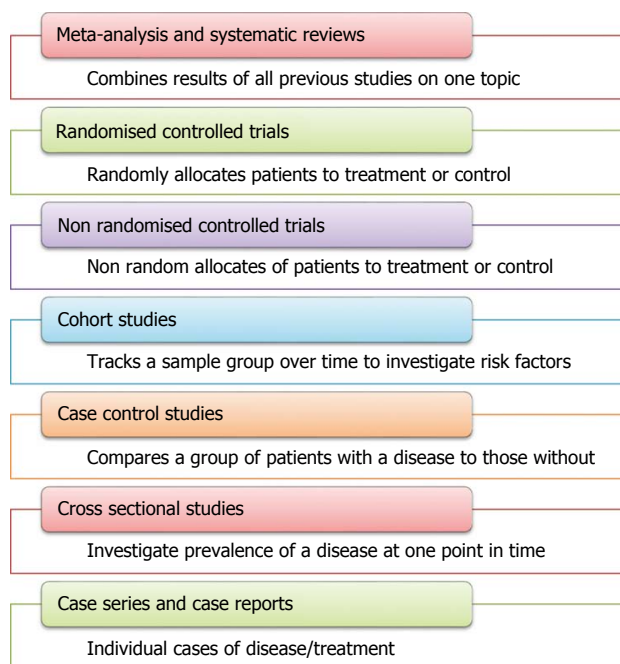


Figure 1 Hierarchy of study design.

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<sup>[5]</sup>. 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<sup>[6]</sup>. 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)<sup>[7]</sup>. CCS also have the ability to report rare infrequent adverse effects, *e.g.*, bile duct injuries in laparoscopic cholecystectomies<sup>[8,9]</sup>. 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<sup>[10]</sup>. Lack of funding and resource constraints have been cited as major obstacles in conducting RCTs<sup>[11]</sup>. Inadequate sample sizes lead to underpowered RCTs which may miss clinically important benefits and lead to type II error<sup>[12]</sup>. Type II error is the failure to reject the null hypothesis when it is false, *i.e.*, False negative results<sup>[12]</sup>.

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<sup>[13]</sup>. 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<sup>[6]</sup>. In addition unlike CCS, RCT also tend to limit the spectrum of disease represented compared to observational studies<sup>[2,6]</sup>.

## 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<sup>[9]</sup>. 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<sup>[14]</sup>. 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<sup>[11]</sup>.

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<sup>[15]</sup>. 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<sup>[7,16]</sup>. However comparisons between results for observational and RCTs in other studies have shown results to be similar between the two in most outcomes<sup>[7]</sup>. 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”<sup>[2,7]</sup>. 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<sup>[2]</sup>.

### 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<sup>[7]</sup>; (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<sup>[17-19]</sup>; (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

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**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.

Garg PK, Singh SK, Prakash G, Jakhetiya A, Pandey D. Role of positron emission tomography-computed tomography in non-small cell lung cancer. *World J Methodol* 2016; 6(1): 105-111 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i1/105.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i1.105>

## 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<sup>[1]</sup>. 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<sup>[2]</sup>. 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

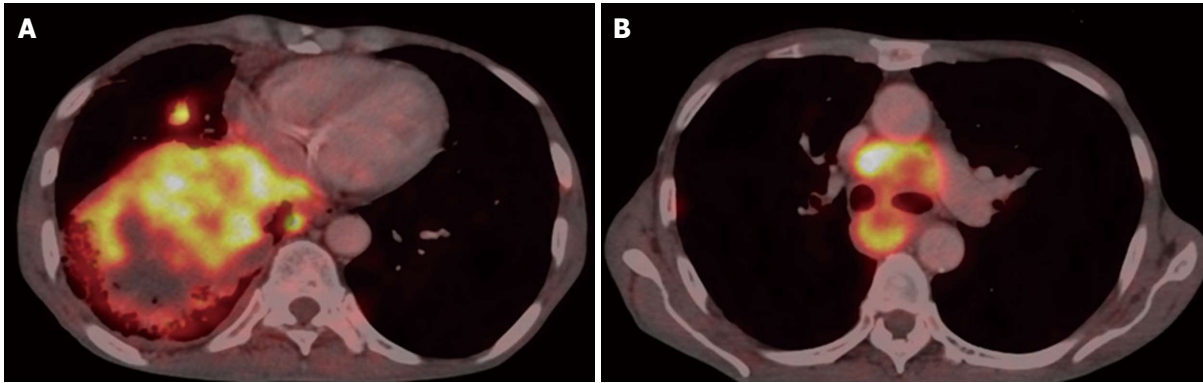
Accurate staging is essential in formulating an optimal management plan for the patient, predicting the prognosis of the disease, and to evaluate and compare the results of various clinical studies by providing a uniform staging terminology across the centers. Staging of NSCLC incorporates assessment 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<sup>[3]</sup>. Pawaroo *et al*<sup>[4]</sup>, 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<sup>[5]</sup>. In an analysis of FDG PET-CT images of 33 lung cancer patients with pleural effusion, Kim *et al*<sup>[6]</sup> 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*<sup>[7]</sup>, 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 thoracentesis may not prove to be futile in up to 30%-40% cases of malignant pleural effusion<sup>[8]</sup>. In malignant pleural effusion, <sup>18</sup>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%<sup>[9]</sup>. Schaffler *et al*<sup>[10]</sup>, evaluated the accuracy of fluorine <sup>18</sup>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), negative 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<sup>[11]</sup>. In a review of three studies including 152 patients total, Toloza *et al.*<sup>[12]</sup> 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.

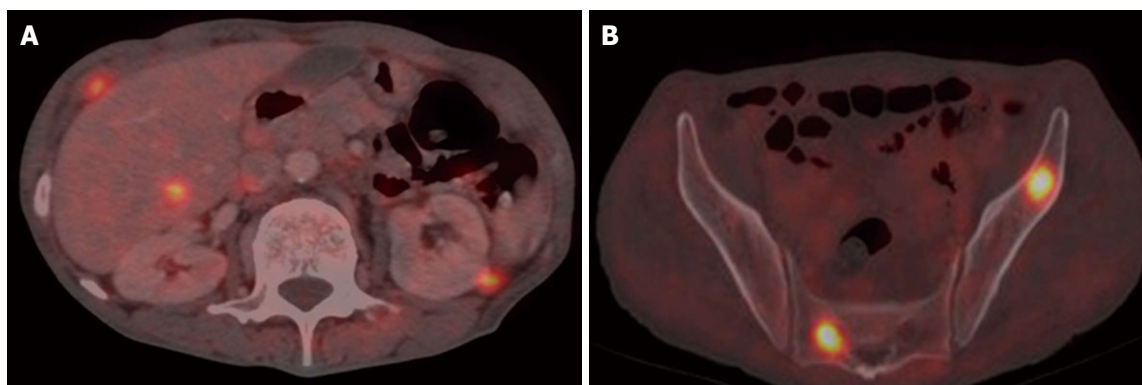
In another study of pathologically proven NSCLC cases who underwent staging using PET/CT and CT from July 2008 to February 2012, Xu *et al.*<sup>[13]</sup> concluded that PET-CT confers significantly higher accuracy than CT in nodal staging. Though PET-CT had a low sensitivity and high false-negative 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.*<sup>[14]</sup> 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.*<sup>[15]</sup> 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<sup>[11]</sup> categorized intrathoracic 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<sup>[3]</sup>. 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<sup>[16]</sup>. 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<sup>[17,18]</sup>.

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<sup>[19]</sup>.

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<sup>[3]</sup>.

Table 1 displays the previously published randomized controlled trials (RCTs) to assess the role of PET in the management of NSCLC<sup>[15,20-23]</sup>. 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 <i>et al</i> <sup>[20]</sup> (PLUS study)	2002	CI $\pm$ brain imaging + invasive diagnostic procedures ( <i>n</i> = 96)	CI $\pm$ brain imaging + PET + invasive diagnostic procedures ( <i>n</i> = 92)	Number of futile thoracotomy	Significant reduction in futile thoracotomy with PET-CT as compared to CI (19 <i>vs</i> 39, <i>P</i> = 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 <i>et al</i> <sup>[22]</sup>	2004	CI ( <i>n</i> = 92)	CI + PET ( <i>n</i> = 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 <i>vs</i> 2, <i>P</i> = 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 <i>et al</i> <sup>[23]</sup>	2006	CI $\pm$ brain imaging + invasive diagnostic procedures ( <i>n</i> = 233)	CI $\pm$ brain imaging + invasive diagnostic procedures ( <i>n</i> = 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) <i>vs</i> 7.9 (1.9), <i>P</i> = 0.90	No significant reduction in total numbers of diagnostic procedures in two groups
Fischer <i>et al</i> <sup>[15]</sup>	2009	CI + invasive diagnostic procedures ( <i>n</i> = 91)	Conventional imaging + PET-CT + invasive diagnostic procedures ( <i>n</i> = 98)	Number of futile thoracotomy	Reduction in futile thoracotomy with PET-CT (21 <i>vs</i> 38, <i>P</i> = 0.05)	PET-CT reduced both the total number of thoracotomies and the number of futile thoracotomies
Maziak <i>et al</i> <sup>[21]</sup>	2009	CI $\pm$ brain imaging + invasive diagnostic procedures ( <i>n</i> = 167)	PET-CT + brain imaging + invasive agnostic procedures ( <i>n</i> = 170)	Correct upstaging to avoid stage inappropriate surgery	Significantly more upstaging with PET-CT as compared to CI (13.8% <i>vs</i> 6.8%, difference 7.0%, <i>P</i> = 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.

III NSCLC 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<sup>[24,25]</sup>. 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<sup>[26]</sup>. 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*<sup>[27]</sup> 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*<sup>[28]</sup> 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*<sup>[29]</sup> 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*<sup>[30]</sup> 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*<sup>[31]</sup> 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.*<sup>[32]</sup> 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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## Potential effects of curcumin on peroxisome proliferator-activated receptor- $\gamma$ *in vitro* and *in vivo*

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### Abstract

Natural peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) 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- $\gamma$ . 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- $\gamma$ .

**Key words:** Curcumin; Anti-inflammatory; Peroxisome proliferator-activated receptor- $\gamma$

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**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- $\gamma$  (PPAR- $\gamma$ ) receptor function and its effects *in vitro* and *in vivo*. Cur affects the

PPAR- $\gamma$  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- $\gamma$  receptor, which leads to the suppression of nuclear factor kappa B, a pro-inflammatory mediator.

Mazidi M, Karimi E, Meydani M, Ghayour-Mobarhan M, Ferns GA. Potential effects of curcumin on peroxisome proliferator-activated receptor- $\gamma$  *in vitro* and *in vivo*. *World J Methodol* 2016; 6(1): 112-117 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i1/112.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i1.112>

## 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<sup>[1-5]</sup>. Cur is a lipophilic polyphenol, is poorly soluble in water and stable at an acidic pH<sup>[6]</sup>. 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<sup>[7]</sup>. Dietary polyphenols have recently received more attention because of their potentially protective characteristics against metabolic diseases<sup>[8]</sup>.

### 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<sup>[9,10]</sup>. 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- $\gamma$  (PPAR- $\gamma$ ) and nuclear factor kappa B (NF- $\kappa$ B)<sup>[11,12]</sup>. It also has a strong anti-inflammatory effect that inhibits several mediators of the inflammatory response<sup>[13-15]</sup>. 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<sup>[16]</sup>.

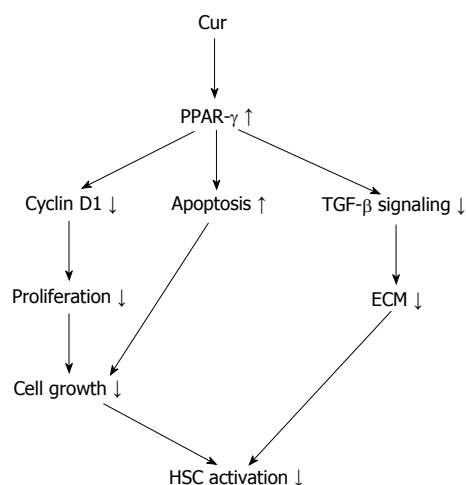
### PPARs

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<sup>[17,18]</sup>. There are 3 major isoforms of PPARs in mammals, namely PPAR $\alpha$ , PPAR- $\gamma$  and PPAR $\alpha/\gamma$ . PPAR- $\alpha$  can improve triglyceride concentration and also has some roles in energy homeostasis, whereas activation of PPAR- $\alpha/\gamma$  improves fatty acid hemostasis<sup>[19]</sup>. PPAR- $\gamma$  is involved in lipid anabolism, adipocyte differentiation inflammation and immune response<sup>[20]</sup>. PPAR- $\alpha$  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- $\gamma$  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- $\gamma$  in systemic metabolic control is due to its pivotal role in the homeostasis control of glucose and lipid homeostasis, lipid storage and adipogenesis<sup>[21]</sup>. Lately, PPAR- $\gamma$  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<sup>[22]</sup>.

### 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<sup>[23,24]</sup>. 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<sup>[25,26]</sup>. Activation of hepatic HSCs is a key step in liver collagen production and fibrosis formation<sup>[27-31]</sup>. 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<sup>[32]</sup>. Some studies showed that HSC activation significantly reduces the expression of PPAR- $\gamma$  and that PPAR- $\gamma$  agonists inhibit HSC activation, resulting in reduced expression of  $\alpha$ -SMA and collagen, as well as reduced cell propagation and development of hepatic fibrosis. In normal liver tissues, PPAR- $\gamma$  is expressed highly in quiescent HSCs. Moreover, increased PPAR- $\gamma$  expression reduces the synthesis of HSC DNA and results in the diminished expression of collagen and the transforming growth factor (TGF)-1 $\beta$ . At the same time, PPAR- $\gamma$  is also involved in the apoptosis of HSCs through a variety of mechanisms<sup>[33-36]</sup>. Some experiments have confirmed that Cur may prevent the proliferation of HSCs whilst also increasing their apoptosis<sup>[37]</sup>. A further study has shown that Cur increases the expression of PPAR- $\gamma$  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- $\gamma$  after modulation with curcumin. PPAR- $\gamma$ : Peroxisome proliferator-activated receptor- $\gamma$ ; HSC: Hepatic stellate cell; TGF: Transforming growth factor; Cur: Curcumin; ECM: Extracellular matrix.

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

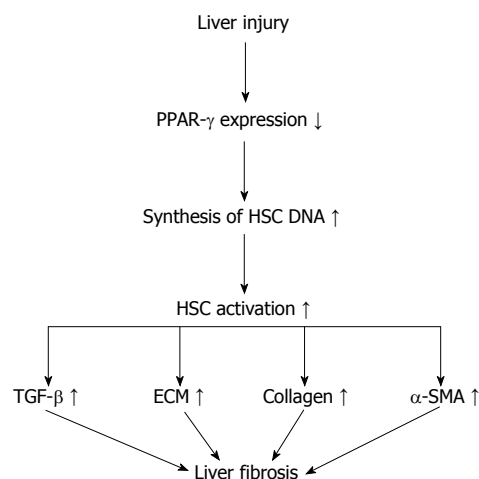
In this review article, we present the current literature to display the role of Cur on modulation of inflammatory processes that are mediated through PPAR- $\gamma$ .

## EFFECTS OF CUR ON PPAR- $\gamma$ 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- $\beta$ <sup>[24,25,39-41]</sup>. During HSC activation, regulatory pathways including epigenetic regulation of (NF- $\kappa$ B) and reduction in PPAR- $\gamma$  expression modulate the expression of many genes, including TGF- $\beta$  and MMP-2<sup>[42-46]</sup>.

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- $\beta$  signaling *via* Cur, including PPAR- $\gamma$  activation. Cur inhibits NF- $\kappa$ B, leptin and insulin and mediates HSC activation by stimulating PPAR- $\gamma$  activity<sup>[38,47-51]</sup> (Figure 2).

Zheng *et al.*<sup>[52]</sup> confirmed that inhibiting PPAR- $\gamma$  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- $\beta$  receptors and disturbed the TGF- $\beta$  signaling pathway in stimulated HSC, which is facilitated by PPAR- $\gamma$  stimulation<sup>[52]</sup>. Zhang



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

*et al.*<sup>[37]</sup> 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- $\gamma$  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- $\gamma$  activation-dependent pathway. Also, other studies showed that PPAR- $\gamma$  could be a target molecule for decreasing pathological angiogenesis in liver fibrosis for rodents<sup>[37]</sup>. These studies offer new perspectives into the mechanisms that underpin prevention of HSC activation by Cur and PPAR- $\gamma$  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.

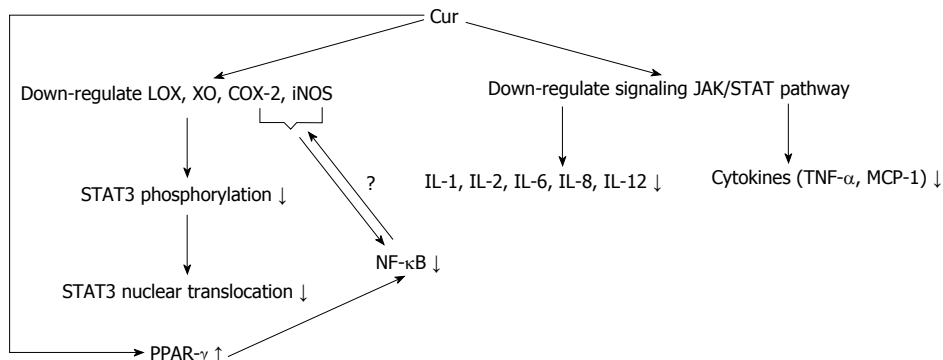
## EVIDENCE FOR THE PPAR- $\gamma$ MEDIATED ANTI-INFLAMMATORY EFFECT OF CUR

It appears that the hydroxyl and methoxy residues of Cur are accountable for its antioxidant and anti-inflammatory effects<sup>[53,54]</sup>. 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- $\gamma$  modulated by curcumin *in vivo* and *in vitro*

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

NF- $\kappa$ B: 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- $\kappa$ B activation and causing its anti-inflammatory effects. Also, Cur with increasing PPAR- $\gamma$  expression directly inhibits NF- $\kappa$ B activation. NF- $\kappa$ B: 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- $\gamma$ : Peroxisome proliferator-activated receptor- $\gamma$ ; 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<sup>[55-58]</sup>. Cur suppression of COX-2 and inducible nitric oxide synthase may be *via* the inhibition of the NF- $\kappa$ B activation by this polyphenol group.

Kawamori *et al.*<sup>[59]</sup> have shown that dietary Cur inhibits phospholipase A2 and affects COX and lipoxygenase actions. Cur decreases COX-2 expression at the transcriptional level<sup>[13]</sup>. Cur is supposed to inhibit NF- $\kappa$ B 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- $\gamma$  agonists found in the diet might be helpful by acting as anti-inflammatory factors<sup>[59-61]</sup>.

Cur has been reported to trigger PPAR- $\gamma$  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- $\gamma$  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- $\gamma$ , or Cur is a ligand of PPAR- $\gamma$  leading to the stimulation of PPAR- $\gamma$ <sup>[62,63]</sup>. A summary of the possible molecular targeting of Cur and PPAR- $\gamma$  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- $\kappa$ B<sup>[4,16]</sup>. Most experiments have shown that the anti-inflammatory effect of Cur is attributed to PPAR- $\gamma$  activation<sup>[64]</sup>. Recent experimental data have shown that Cur has an antitumor effect in pancreatic cancer by inhibiting propagation and down-regulating NF- $\kappa$ B and its products<sup>[65]</sup>. Nevertheless, it is reasonable to suggest that Cur prompted an anti-inflammatory effect through the up-regulation of PPAR- $\gamma$  which is closely related to the NF- $\kappa$ B 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- $\gamma$  receptor function. Cur prompts the expression of the PPAR- $\gamma$  gene, causing its activation in cells to activate HSCs and hepatic fibrosis. This combined action of Cur and PPAR- $\gamma$  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- $\gamma$  receptor, which leads to the suppression of NF- $\kappa$ B, 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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**Institutional review board statement:** This study was reviewed and approved by the Ethics and Research Advisory Committee of the National Tuberculosis Control Programme, Ministry of Health, Ebonyi State, Nigeria.

**Informed consent statement:** As this was a retrospective study, the consent of the patients was not obtained, however, patient records was anonymized and de-identified prior to analysis.

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

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**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<sup>[1]</sup>. Adverse outcomes of TB tend to be high in poor populations<sup>[2]</sup>. 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<sup>[2]</sup>. Rural residence can be a marker for poverty and, thus vulnerability to TB<sup>[3]</sup>. Previous studies demonstrate that rural TB patients often do not recognise TB symptoms<sup>[4]</sup>. This have resulted in prolonged delays in seeking care for TB<sup>[4,5]</sup>, and may lead to increased risk of continuing community TB transmission and important burden of undiagnosed active TB in the community<sup>[6]</sup>. Moreover, when these rural patients eventually present to health services, they arrive with more advanced TB disease<sup>[7]</sup>. 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<sup>[8]</sup>. 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<sup>[2,3,5]</sup>.

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<sup>[9]</sup>, 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<sup>[1,10]</sup>. Furthermore, identifying the specific determinants for unsuccessful outcomes is important to design interventions that would improve treatment systems<sup>[9,11]</sup>. As addressing the needs of TB among the poor and vulnerable populations is one of the key components of the StopTB strategy<sup>[12]</sup>, 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<sup>[10,13-17]</sup>, and far less have documented independent risk factors for poor treatment outcomes among them in high-burden settings<sup>[15,16]</sup>.

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<sup>[18,19]</sup>. Also, a high proportion of rural TB patients in Nigeria face substantial patient and health systems delays before reaching an appropriate health care provider<sup>[5]</sup>. 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<sup>[19,20]</sup>. Thus, there is a need to assess the performance of the tuberculosis control programme in rural Nigeria in order to 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<sup>[21-23]</sup>. The present study population consisted of all adult ( $\geq 15$  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<sup>[18]</sup>; and 74% live below the poverty line<sup>[24]</sup>. TB notification rate for new cases was 77/100000 in 2009<sup>[25]</sup>. Due to health system gaps, there is limited availability of public facilities in rural settings in the state<sup>[19,20]</sup>. 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<sup>[25]</sup>; 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)<sup>[26]</sup>. 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<sup>[26]</sup>. 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<sup>[26]</sup>.

### 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<sup>[26,27]</sup>. 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<sup>[27]</sup>. 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<sup>[26,27]</sup>. 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<sup>[26]</sup>.

### Definitions of TB treatment outcomes

We used the standard WHO definitions of TB treatment outcomes<sup>[1,26,27]</sup>. 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 Episcopy 2.0. With a sample size of at least 246 patients, we were able to detect an 80% prevalence of successful outcomes<sup>[28]</sup>, 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  $\pm$  SD age of all patients was  $39.3 \pm 15.1$  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+ <i>n</i> (%)	HIV-negative <i>n</i> (%)	HIV-positive <i>n</i> (%)	$\chi^2$ ( <i>P</i> 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) <sup>1</sup>
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) <sup>1</sup>
Default	12 (26.1)	10 (26.3)	2 (25.0)	0.0 (0.68) <sup>1</sup>
Transfer-out	8 (17.4)	7 (18.4)	1 (12.5)	0.07 (0.64) <sup>1</sup>
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) <sup>1</sup>
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) <sup>1</sup>
Death	11 (13.6)	8 (12.3)	3 (18.8)	0.75 (0.31) <sup>1</sup>
Default	3 (3.7)	3 (4.6)	0 (0)	0.58 (0.59) <sup>1</sup>
Transfer-out	3 (3.7)	1 (1.5)	2 (12.5)	4.74 (0.08) <sup>1</sup>
Total	81	65	16	

<sup>1</sup>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%<sup>[1]</sup>. By 2011, both Nigeria and Ebonyi State had reached the WHO target treatment success rate of 85% among all TB patients<sup>[1,28]</sup>. 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%)<sup>[13]</sup>, China (74.5%)<sup>[14]</sup> and Ghana (60.7%)<sup>[29]</sup> 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%<sup>[10,16,29]</sup>. 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	<i>n</i> = 1180 <i>n</i> (%)	Unsuccessful outcomes <i>n</i> (%)	Crude OR (95%CI)	Adjusted OR (95%CI)	Adjusted <i>P</i> -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 ( <i>n</i> = 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<sup>[17,30]</sup>. 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<sup>[5,17,19,30]</sup>. 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<sup>[1,31,32]</sup>, 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<sup>[1,31]</sup>. Also, lack of cavitory lesions in their lungs makes them prone to misdiagnosis, delayed diagnosis, and higher co-morbidities often resulting in poor outcome<sup>[1,31,32]</sup>. Also, as previously documented<sup>[31,32]</sup>, 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<sup>[31]</sup>. 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<sup>[2,19,20]</sup>, however, this needs to be con-

firmed 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<sup>[33]</sup>. 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<sup>[27]</sup>. 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<sup>[13,15-17,31]</sup>. 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<sup>+</sup> 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.

## ACKNOWLEDGMENTS

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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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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

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**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<sup>[1,2]</sup>. However, nebulizers are potential sources of microbial contamination of the respiratory tract<sup>[3,4]</sup>. Small-volume medication nebulizers for administering bronchodilators, including hand-held nebulizers, can produce bacterial aerosols<sup>[5]</sup>. Both jet and ultrasonic hand-held nebulizers have been associated with nosocomial pneumonia<sup>[6-9]</sup>.

The Centers for Disease Control and Prevention (CDC) established guidelines for preventing nosocomial pneumonia in 1997<sup>[10]</sup>. 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<sup>[11]</sup>. 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<sup>[12]</sup>. 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.



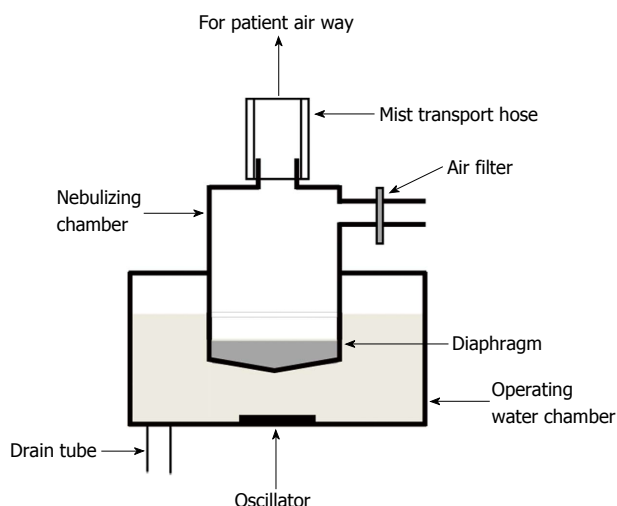


Figure 1 Components and structure of an ultrasonic nebulizer.

### 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<sup>[11]</sup>. 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).

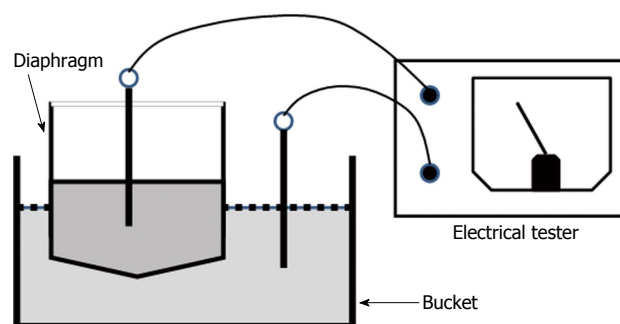


Figure 2 Scheme for discovering diaphragm damage using an electrical tester. A low concentration of detergent is added to the water in the diaphragm and bucket. Diaphragm breakage or pinholes are detected by measuring the electricity between the diaphragm and bucket.

### 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 °C 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<sup>[12]</sup>. 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															Total
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	
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.

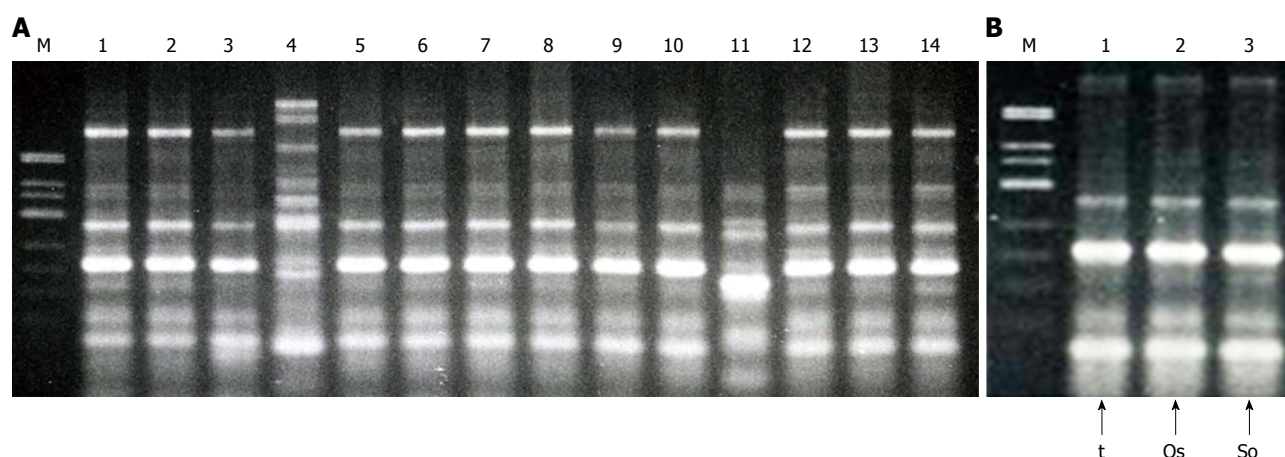
#### **Genotypic analysis of Bcc isolates**

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)<sup>[12]</sup>. 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<sup>[13]</sup>. 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<sup>[14-17]</sup>. 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<sup>[4]</sup>. 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<sup>[18]</sup>. 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<sup>[19]</sup>. Jang *et al.*<sup>[20]</sup> 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<sup>[21,22]</sup>. 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.

## ACKNOWLEDGMENTS

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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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## Methods, units and quality requirements for the analysis of haemoglobin A<sub>1c</sub> in diabetes mellitus

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### Abstract

The formation of glycohemoglobin, especially the hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) fraction, occurs when glucose becomes coupled with the amino acid valine in the  $\beta$ -chain of Hb; this reaction is dependent on the plasma concentration of glucose. Since the early 1970s it has been known that diabetics display higher values of HbA<sub>1c</sub> because they have elevated blood glucose concentrations. Thus HbA<sub>1c</sub> has acquired a very important role in the treatment and diagnosis of diabetes mellitus. After the introduction of the first quantitative measurement of HbA<sub>1c</sub>, numerous methods for glycohemoglobin have been introduced with different assay principles: From a simple mini-column technique to the very accurate automated high-pressure chromatography and lastly to many automated immunochemical or enzymatic assays. In early days, the results of the quality control reports for HbA<sub>1c</sub> varied extensively between laboratories, therefore in United States and Canada working groups (WG) of the Diabetes Controls and Complications Trial (DCCT) were set up to standardize the HbA<sub>1c</sub> assays against the DCCT/National Glycohemoglobin Standardization Program reference method based on liquid chromatography. In the 1990s, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) appointed a new WG to plan a reference preparation and method for the HbA<sub>1c</sub> measurement. When the reference procedures

were established, in 2004 IFCC recommended that all manufacturers for equipment used in HbA<sub>1c</sub> assays should calibrate their methods to their proposals. This led to an improvement in the coefficient of variation (CV%) associated with the assay. In this review, we describe the glycation of Hb, methods, standardization of the HbA<sub>1c</sub> assays, analytical problems, problems with the units in which HbA<sub>1c</sub> values are expressed, reference values, quality control aspects, target requirements for HbA<sub>1c</sub>, and the relationship of the plasma glucose values to HbA<sub>1c</sub> concentrations. We also note that the acceptance of the mmol/mol system for HbA<sub>1c</sub> as recommended by IFCC, *i.e.*, the new unit and reference ranges, are becoming only slowly accepted outside of Europe where it seems that expressing HbA<sub>1c</sub> values either only in per cent units or with parallel reporting of percent and mmol/mol will continue. We believe that these issues should be resolved in the future and that it would avoid confusion if mmol/mol unit for HbA<sub>1c</sub> were to gain worldwide acceptance.

**Key words:** Diabetes; Hemoglobin A<sub>1c</sub>; Glycohemoglobin; Glucose; International Federation of Clinical Chemistry and Laboratory Medicine; Reference values; Quality assurance; Recommendation; Target limits

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**Core tip:** The aim of this review is to clarify methods, units, quality requirements, reference and cutoff limits for hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) and ratio of blood glucose/HbA<sub>1c</sub> on the basis of the results from Finnish quality control surveys by comparing them to the literature. The HbA<sub>1c</sub> surveys of Labquality Ltd. (Helsinki, Finland) were started in 1986 by using two fresh EDTA-blood samples. From 1994, the number of the participating laboratories had risen to 139, of which 75 were Finnish and 64 from five other countries. In 2014, the number of the participating laboratories was total 214, 141 were Finnish and 73 from 13 other countries.

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## INTRODUCTION

Fisher<sup>[1]</sup> synthesized a molecule named fructosamine in 1889. In the 1950s and 60s, it was reported that carbohydrate residues could become attached to hemoglobin (Hb)<sup>[2,3]</sup>. In 1967 Holmquist and Schroeder<sup>[4]</sup> observed that glucose bound to Hb and Rahbar<sup>[5]</sup> revealed that the electrophoretic fraction containing glucose was higher in blood samples from diabetic

subjects than in healthy subjects. Subsequently, Shapiro *et al*<sup>[6]</sup> described that many carbohydrate components including glucose could be bound to Hb and that glucose was most efficiently bound to the β-chain of Hb. After Trivelli *et al*<sup>[7]</sup> published their quantitative measurement of Hb fractions in human blood, the analysis of HbA<sub>1c</sub> was recognized as being a very important parameter in the assessment of diabetic patients.

In the early years, the results of HbA<sub>1c</sub> analysis varied extensively between methods and laboratories<sup>[8-12]</sup>. Therefore during the 1980s, in United States and Canada working groups (WG) of the Diabetes Controls and Complications Trial (DCCT) were set up; these were originally incorporated within a multicenter, randomized clinical trial designed to compare treatments of insulin dependent diabetes mellitus in the National Glycohemoglobin Standardization Program (NGSP), but subsequently this activity was expanded to standardize the HbA<sub>1c</sub> assays to the DCCT/NGSP reference method of liquid chromatography<sup>[9]</sup>.

Later in the 1990s, the International Federation of Clinical Chemistry (IFCC) decided to develop a reference system for the international standardization of HbA<sub>1c</sub>/glycohemoglobin measurements<sup>[13]</sup>, which became the basis of the reference laboratory network for HbA<sub>1c</sub>. In addition, IFCC set up another WG on Standardization for HbA<sub>1c</sub> (1994/1995) in order to develop a primary standard<sup>[14]</sup> and reference method<sup>[15]</sup> for worldwide use in the HbA<sub>1c</sub> measurement. These were the reasons why from the 1990s, IFCC organized WG to promote the standardization of all types of assays for HbA<sub>1c</sub>. The adoption of these recommendations achieved substantial improvements in the analytical aspects and clinical significance of HbA<sub>1c</sub><sup>[16,17]</sup> so that today reference standards and standardized methods are in widespread use.

Further in 2010, the American Diabetes Association (ADA) emphasized the important role of HbA<sub>1c</sub> in the diagnosis of diabetes, setting an analytical cutoff limit value of 6.5% for HbA<sub>1c</sub> values<sup>[18]</sup> if they were expressed in percentage terms. Recently Hanas and John<sup>[19]</sup> reported the conclusions of the International Consensus Committee 2013 Update that HbA<sub>1c</sub> results should be reported by clinical laboratories worldwide in Système Internationale (SI) units (mmol/mol - no decimals) and the corresponding NGSP units (% - one decimal) and recommended strongly to the editors of scientific journals that submitted manuscripts should report HbA<sub>1c</sub> values in both SI (IFCC) and NGSP/DCCT units.

This review provides a brief summary of the reaction of glucose with Hb and the possible techniques available for analyzing HbA<sub>1c</sub>. It also reviews the quality control, requirements of the target limits and considers issues related to the units and cutoff limits for HbA<sub>1c</sub> in relation to the recommendations of IFCC and ADA.

## GLYCATION OF Hb

Glucose is the major soluble carbohydrate and it can combine with different protein molecules in blood and



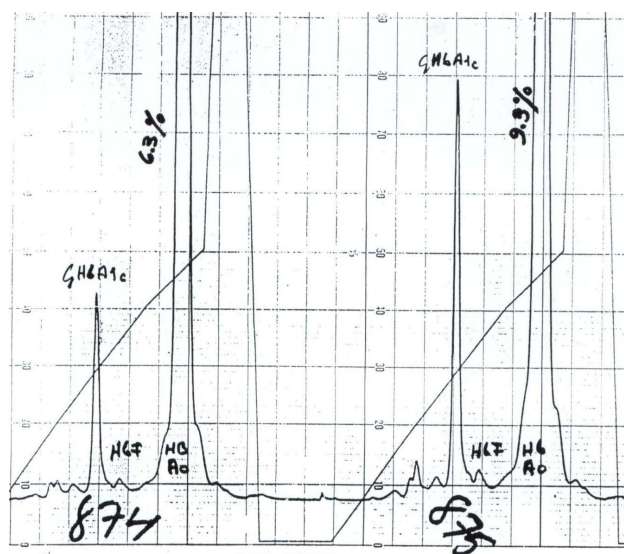


Figure 1 Fractionation of blood hemoglobin by an automated Mono S™ HR5/5 cation exchange column from Pharmacia with malonate buffer (0.01 mol/L, pH 5.7, 11 min, room temperature) for normal and diabetic human blood samples is illustrated as a chromatogram (Penttilä *et al.*<sup>[10]</sup>).

tissues depending on the glucose concentration. In blood, Hb is one of these proteins and the  $\beta$ -chain of Hb is the main target of glucose<sup>[6]</sup>. Glucose is initially non-enzymatically bound to the amino acid valine on the  $\beta$ -chain of Hb *via* the formation of a reversible aldimine moiety, which then becomes rearranged into the irreversible ketoamine form. The structure of this ketoamine is similar to that of fructosamine<sup>[6]</sup>. This phenomenon occurs during the whole lifetime of erythrocytes (120 d) and thus the content of the ketoamine in Hb correlates with the age of the erythrocytes and the value of HbA<sub>1c</sub> normally represents a mean value reflecting a time period starting from about three months before sampling.

By analyzing Hb components in blood, Trivelli *et al.*<sup>[7]</sup> demonstrated that the small fraction A<sub>1c</sub> was glycated more than the other small fractions A<sub>1a</sub> and A<sub>1b</sub> and that the amounts of this A<sub>1c</sub> fraction were clearly higher in the blood of diabetic patients than in the normal subjects. The fractionation of Hb by a Mono S™ HR5/5 cation exchange column from Pharmacia with 0.01 mol/L malonate buffer for normal and diabetic human blood samples is illustrated in Figure 1<sup>[20]</sup>.

## METHODS OF HbA<sub>1c</sub> ASSAYS

The first methods for the HbA<sub>1c</sub> measurements were simple electrophoretic or ion-exchange mini-column chromatography assays, but they were rather soon replaced by many automated techniques, *e.g.*, different liquid chromatography techniques [ion-exchange chromatography (HPLC or FPLC) or affinity chromatography]<sup>[8-12,20-22]</sup> as seen in Tables 1 and 2. The NGSP/DCCT organizations in United States and Canada selected the automated Bio-Rex 70 liquid

Table 1 The methods commonly used for analyzing hemoglobin A<sub>1c</sub> according to the surveys conducted by Labquality Ltd. in 1994

Numerical summary from a diabetic EDTA-blood sample 1/1994				
Analyte/method group	Mean	SD	CV%	Number
GHbA <sub>1c</sub> %				
Ion exchange Ciba Corning	8.7	0.4	4.3	4
Ion exchange Diamat Biorad	9.7	0.8	8.4	15
Ion exchange Kyoto Daichii	8.4	0.3	3.4	3
Ion exchange Pharmacia MonoS	9.0	0.8	8.7	17
Ion exchange Shimadzu	10.7	0.1	0.7	2
Ion exchange others	9.5	0.8	7.9	5
Ion exchange minicolumn Biorad	9.7	0.8	8.1	4
Electrophoresis Beckman	9.7	0.8	7.8	8
Affinity method Abbott Imx	8.4	0.6	7.5	15
Affinity method Abbott Vision	8.8	0.4	4.8	8
LainIA Ames DCA 2000	9.1	0.2	2.6	29
TinIA Boehringer	9.1	1.0	10.4	15
Immunochemical others	8.8	1.7	19.3	2
All method groups	9.2	0.8	8.9	127
GHbA <sub>1</sub>				
Ion exchange Ciba Corning	11.8	0.3	2.4	2
Ion exchange Diamat Bio-Rad	12.1	0.4	3.2	7
Ion exchange others	11.5	-	-	1
All methods groups	12.0	0.4	3.3	10
GHb				
Affinity method Abbott Imx	13.4	0.4	2.7	2
Affinity method mini col Isolab	10.3	1.4	13.7	2
All method groups	11.8	2.0	16.5	4

HbA<sub>1c</sub>: Hemoglobin A<sub>1c</sub>.

chromatography assay as the reference method for the HbA<sub>1c</sub> measurement<sup>[9]</sup>. It should be stressed that this equipment has not been available for many years. However, in addition to the NGSP/DCCT system, there were other different assay techniques used for standardization and these had their own standards, *e.g.*, in Japan (NGH) and in Sweden (FPLC with a Mono S column). These methods could be compared to each other and to the new IFCC procedure by using the appropriate master equations<sup>[23]</sup>.

In 1988, an automated immunoassay method for the epitope assays of proteins was developed<sup>[24]</sup> and then utilized in the assay of HbA<sub>1c</sub>. These immunoassay or enzymatic methods have replaced the chromatography methods previously used in clinical laboratories so that today only about 20% to 40% of methods for HbA<sub>1c</sub> are based on liquid chromatographic techniques<sup>[16,25]</sup>. The numbers of immunoassays for HbA<sub>1c</sub> based on many different principles are continuously increasing but these techniques usually have a higher inter-laboratory CV% than can be attained with liquid chromatography or enzymatic methods<sup>[16,22,25-27]</sup>; some of the most important immunoassay methods are listed in Tables 1 and 2.

## STANDARDIZATION OF HbA<sub>1c</sub>

As noted before, there were marked inter-laboratory differences in the quality assurance results before standardization procedures were adopted<sup>[8-12]</sup>. It was

**Table 2** The methods commonly used for analyzing hemoglobin A<sub>1c</sub> according to the surveys conducted by Labquality Ltd. in 2015

Numerical summary from a diabetic EDTA-blood sample 5/2015						
Analyte/method group	Mean	SD	CV%	Min	Max	Number
HbA <sub>1c</sub> , mmol/mol						
Abbott Arhitect enzymatic	53.64	2.45	4.6	48.4	32.1	6
Axis-Shield Afinion	54.70	2.17	4.0	51.0	58.0	44
Beckman Coulter	55.2					1
Hemoque	51.4	3.98	7.7	46.0	55.0	5
HPLC Bio-Rad D-10	55.00					1
HPLC Bio-Rad Variant	56.00	3.21	5.7	54.0	58.0	2
HPLC Tosoh	56.42	1.23	2.2	35.6	59.8	33
Roche Cobas c Tina-Quant	54.76	2.56	4.7	33.0	57.0	13
Roche Cobas Integra	55.0					1
Rocgw Tina Quant	56.00					1
Siemens Advia Centaur	57.93					1
Siemens DCA 2000+ and Vant	55.05	1.96	3.6	51.0	58.0	33
Thermo Scientific Konelab	54.44	2.63	4.8	48.0	58.4	24
All	55.05	2.17	3.9	32.1	59.8	165
HbA <sub>1c</sub>						
Abbott Arhitect enzymatic	7.20	0.00	0.0	7.2	7.2	3
Axis-Shield Afinion	7.17	0.21	2.9	6.9	7.5	44
Beckman Coulter	7.2					1
Hemoque	6.88	0.25	3.7	6.6	7.2	6
HPLC Bio-Rad D-10	7.2					1
HPLC Bio-Rad Variant	7.5					1
HPLC Tosoh	7.34	0.13	1.8	7.0	8.8	23
Roche Cobas c Tina-Quant	7.29	0.12	1.6	6.7	7.4	7
Siemens Advia Centaur	7.45			5.8	7.0	1
Siemens DCA 2000+ and Vant	7.19	0.18	2.6	6.8	7.5	31
Thermo Scientific Konelab	7.13	0.25	3.5	6.6	7.5	26
All	7.20	0.21	2.9	6.6	8.8	143

HbA<sub>1c</sub>: Hemoglobin A<sub>1c</sub>.

also reported that by incorporating an extra sample, against which the primary results could be recalculated, and significantly smaller CV% values could be obtained<sup>[9,16,17]</sup>.

After the appearance of the IFCC WG for HbA<sub>1c</sub>, a reference system was soon organized in 1996<sup>[13]</sup>, with the goal of achieving standardization of HbA<sub>1c</sub> analysis; this formed the basis for the worldwide reference laboratory network to help clinical laboratories in their HbA<sub>1c</sub> measurements. The reference preparations which represented the primary and secondary standards of HbA<sub>1c</sub> and HbA<sub>0</sub> were produced in 1998<sup>[14]</sup> followed in 2002 by the reference method for the specific measurement of HbA<sub>1c</sub><sup>[15]</sup>. The final measurements in the reference method were based on the assay of N1-deoxyfructosyl-hemoglobin<sup>[28]</sup>. The assay consisted of a primary fractionation of the sample by affinity chromatography followed by analysis utilizing either HPLC/electrospray mass spectrometry or HPLC/capillary electrophoresis. The primary and secondary standards and the reference methods and the guidelines about names and units were then introduced to be adopted worldwide by all manufacturers making equipment for analysis of HbA<sub>1c</sub> and also for clinical laboratories<sup>[13,23,25,29]</sup>.

The standardization protocols about HbA<sub>1c</sub> have been published by many societies of laboratory medicine in

their own languages, e.g., ADA in United States<sup>[18,29]</sup>, DGKL in Germany<sup>[30]</sup>, NEQAS in United Kingdom<sup>[31]</sup>, SiBioC in Italy<sup>[32]</sup>, EQUALIS in Sweden<sup>[33]</sup>, Finnish Society of Clinical Chemistry (FSCC) in Finland<sup>[34]</sup>, etc. With respect to the standardization procedures, it should be noted that according to Weykamp *et al.*<sup>[35]</sup> the units and standardization protocols for the NGSP/DCCT and IFCC procedures are different for NGSP/DCCT and IFCC results and thus the reference values and quality control requirements cannot be of the same size.

## ANALYTICAL PROBLEMS OF HbA<sub>1c</sub> ASSAYS

The typical problems<sup>[36]</sup> which interfere with the HbA<sub>1c</sub> assays, are attributable to hyperbilirubinemia, hypertriglyceridemia, leukocytosis and many Hb variants. In addition, certain physiological and pathological characteristics such as gestational stage, age, race, pre-symptomatic type 1 diabetes, malaria, iron deficiency, bleedings, transfusions, splenectomy, kidney failure, alcohol abuse, and some drugs may affect the HbA<sub>1c</sub> results. The HbA<sub>1c</sub> results may be too high in some cases and too low in others (hemolysis, pregnancy).

If erythrocytes have a short life-time, e.g., in hemolytic anemias, this may decrease the HbA<sub>1c</sub> results. Some abnormal forms of Hb cause erroneous results

**Table 3** Summary of the questionnaires about the use of the hemoglobin A<sub>1c</sub> units sent to 35-51 societies of laboratory medicine (mainly clinical chemistry) and returned by 28.12.2014

Year	2009	2011	2013	2014
Queries sent	35	37	47	51
Only % unit	16	14	15	17
Parallel units, % and mmol/mol	4	9	12	11
mmol/mol unit only in use	( <sup>1</sup> )	6	11	13
No reply	15	8	9	10

<sup>1</sup>Germany was the first country which adopted the exclusive use of mmol/mol unit for HbA<sub>1c</sub> from 1.1.2010. The questionnaires have been dispatched by e-mail, telefax and mail mainly to Europe and to a small extent outside of Europe from 2009 to 2014.

(HbF, HbS, HbC, HbD, HbE, etc.) depending on the assay type<sup>[37-39]</sup>. Furthermore, iron deficiency anemia as well as iron deficiency without anemia may induce elevated HbA<sub>1c</sub> values compared with controls even though blood glucose levels are normal<sup>[39]</sup>.

Many of these errors can be very difficult to detect, especially when using immunochemical assays. With some systems such as with liquid chromatography, the erroneous results can be visualized in the chromatograms, and these assays are commonly used for comparison<sup>[23,35-40]</sup>. However, errors can also be due to the problems in the action or response of insulin which are not directly related to the methods being used to assay HbA<sub>1c</sub><sup>[36]</sup>.

## UNITS OF HbA<sub>1c</sub> IN LABORATORY PRACTICE

In 2009, prior to the use of the new IFCC system with the accepted name and unit for HbA<sub>1c</sub>, questionnaires about which units should be used were sent to the European societies of laboratory medicine and some other societies outside of Europe (mainly in clinical chemistry)<sup>[40]</sup>. Germany was the first country to adopt mmol/mol exclusively in the daily laboratory practice (Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriums-medicin); this was inaugurated at the start of 1.1.2010. Germany was followed later in 2011 by The Netherlands, Sweden and the United Kingdom. Then gradually the number of mmol/mol reports of HbA<sub>1c</sub> increased up to 13 in 2014, representing 25% of all replies from 51 queries. During these years, there was also an increase in the numbers of laboratories (from 9 to 12) reporting HbA<sub>1c</sub> values in parallel units, *i.e.*, in both % and mmol/mol. By 2014, a minority of responders (24%) stated that they were using only the mmol/mol whereas nearly every other respondent (49%) stated that values were being expressed not only in mmol/mol but also as % values (Table 3). However, by the end of 2014, ten societies had not responded to the questionnaire, although the e-mail, telefax and mail addresses were taken from the

annual catalogues of IFCC.

On the other hand, in the period from 2010 to 2015, the users of % values in the HbA<sub>1c</sub> surveys conducted by Labquality Ltd.<sup>[41]</sup> have gradually but significantly decreased (from 54% to 40%).

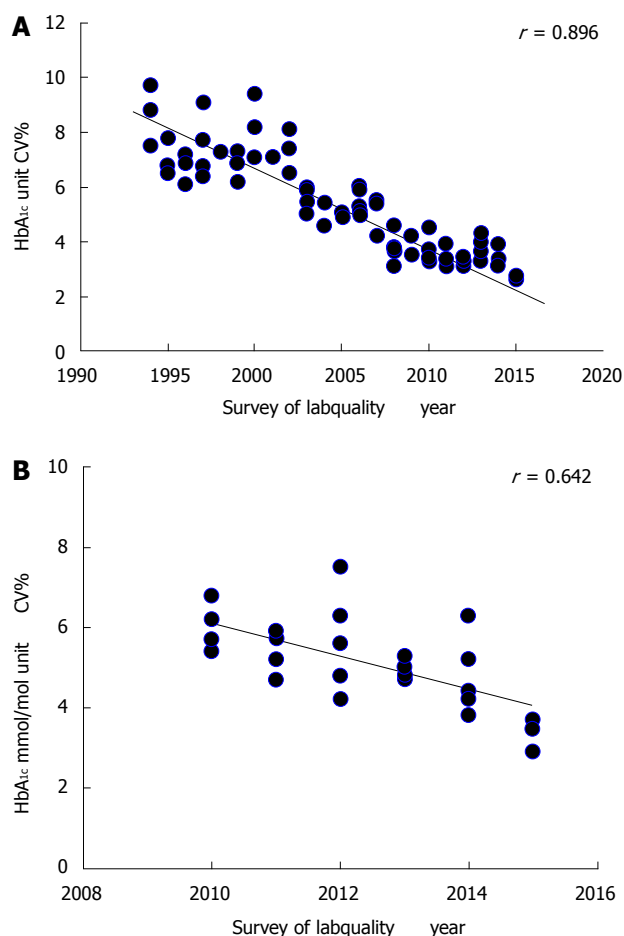
The Finnish experience is an example of the difficulty to obtain acceptance of the molar unit recommended by IFCC for expressing HbA<sub>1c</sub>. In 2009, the FSCC recommended that clinical laboratories should report the HbA<sub>1c</sub> results in parallel, *i.e.*, as mmol/mol and % values according to the recommendations<sup>[23]</sup>. In 2011, the FSCC proposed that the HbA<sub>1c</sub> values should be provided only in mmol/mol. Nonetheless, the issue has not fully resolved due to the opposition from physicians treating diabetics. Thus the following compromise was agreed: In the future, the laboratories will report the HbA<sub>1c</sub> values in parallel units, *i.e.*, as mmol/mol and % although the measurements in many laboratories have originally been calculated in mmol/mol<sup>[40]</sup>.

In Finland, one out of five university hospital districts (Pirkanmaa and Kanta-Häme) stopped reporting the % values for HbA<sub>1c</sub> from April 2014; in that hospital the HbA<sub>1c</sub> values are only being reported in mmol/mol<sup>[40]</sup>. It is possible that some other Finnish hospital districts will come to the same decision in the near future. In September 2014, the board of FSCC established a new working group to discuss with physicians and with non-laboratory societies and as well as with other interested parties about the issues related to the determination of HbA<sub>1c</sub>. During the summer of 2015, FSCC decided to recommend that from 1.1.2016 the HbA<sub>1c</sub> values should no longer be expressed as % units; hopefully this recommendation will prove acceptable.

## QUALITY ASSURANCE AND TARGET LIMITS OF HbA<sub>1c</sub>

The QC surveys utilize common statistical methods such as the coefficient of variation (CV%)<sup>[16-18]</sup>. On the basis of these and the standard deviation (SD) values, the new target limits have been calculated for the QC-surveys of HbA<sub>1c</sub> around the mean and the reference values expressed as either % or mmol/mol values. This means that within the range mean  $\pm$  2SD 95.6% of all results are within the acceptable limits and these correspond to the recent findings of Hytöft Petersen and Lee<sup>[42]</sup>. Furthermore, the new report of Nielsen *et al.*<sup>[43]</sup> about the value of HbA<sub>1c</sub> in the classification of diabetes highlights the importance of the exact and precise measurement of HbA<sub>1c</sub>.

As mentioned earlier, there was a remarkably high variation in the values being obtained with the different methods being used in laboratories all around the world<sup>[8-12]</sup>. Weykamp *et al.*<sup>[11]</sup> were one of the first groups who described the dramatic improvement that could be obtained in the CV% of the HbA<sub>1c</sub> results of either normal or diabetic subjects when the primary results were recalculated by incorporating an extra sample with



**Figure 2** The total variations of the glycohemoglobin results have been presented both in % and in mmol/mol units from the surveys of Labquality Ltd. from 1994 to 2015. In the part A have been presented the total variations of the glycohemoglobin in % units and in the part B the total variations of HbA<sub>1c</sub> in mmol/mol units.

a known HbA<sub>1c</sub> value. They had reviewed the corrected results from 110 laboratories using 21 different methods.

In Finland, the glycohemoglobin quality assurance surveys of Labquality started in 1986 with two fresh native EDTA-blood samples, one at the normal level and the second at the diabetic level, which were sent in each survey to all participants<sup>[41]</sup>. The mean values of the surveys were used as the target values and the acceptable ranges were  $\pm 10\%$  around the mean values. Figure 2A displays the significant improvement in the HbA<sub>1c</sub> % values ( $r = 0.896$ ) from 1994 to 2015 expressed as CV% values, they correspond well to the earlier publications<sup>[16,17]</sup>. Similarly in Figure 2B from 2009 when the results were reported in mmol/mol units, a further improvement in the CV% was observed (for results expressed as mmol/mol) ( $r = 0.642$ ). The mean  $\pm$  SD values and CV% have been calculated from the surveys of Labquality.

In the most recent survey (2015) the range mean  $\pm$  2SD indicates that 95% of all results are within these limits<sup>[9,44,45]</sup>. For example, in December 2014, at the HbA<sub>1c</sub> level of 6.77%, near the cut-off limit of ADA of

6.5%<sup>[18]</sup>, the SD value was 0.21 for % units and that for mmol/mol units at the level of 50.8 mmol/mol the corresponding SD value was 2.1 mmol/mol. Thus the calculated acceptable limits with the old range of mean HbA<sub>1c</sub> 6.77%  $\pm$  10% were from 6.09% to 7.48% and with the new narrower limit dating from 1.1.2015 the limits with mean  $\pm$  6% were from 6.36% to 7.18%. The latter range corresponds well with that of 6.77%  $\pm$  0.21% with the CV of 3.26% for the survey ( $0.21/6.77 \times 100\%$ ). If one takes the mean  $\pm$  2SD of all results, then the range would be 6.35% to 7.19% in that survey and these would be clearly inside the old range of 10% and similar to the new limits from Labquality. Correspondingly in the same survey of December 2014 for mmol/mol values, the mean HbA<sub>1c</sub> value was  $50.8 \pm 2.1$  mmol/mol and the range for mean  $\pm$  2SD extended from 46.6 to 55.0 mmol/mol being very close to the new mmol/mol limit from Labquality as mean  $\pm$  8%, i.e., from 46.7 to 54.9 mmol/mol with the CV% of 4.13% ( $2.1/50.8 \times 100\%$ ). In the future, the target limits for HbA<sub>1c</sub> of Labquality may be made more demanding. The latest Finnish results that in 2015 the CV% values for HbA<sub>1c</sub> have reached a sufficiently low level (Figure 2) to be comparable to others in the literature both for % and mmol/mol results<sup>[9,16-18,46,47]</sup>.

From the survey conducted in 2002, the HbA<sub>1c</sub> values of the EDTA blood samples for the % results were also analyzed by the European Reference Laboratory for Glycohemoglobin (ERLGH)<sup>[48]</sup>. It was found that there was an almost perfect correlation between the mean HbA<sub>1c</sub> % values of Labquality and the values HbA<sub>1c</sub> % values of ERLGH ( $r = 0.997$ ). The same phenomenon was also seen for the mmol/mol results from 2010 when comparing the mean values of Labquality and those of ERLGH ( $r = 0.973$ ). Since the mean values of the surveys conducted by Labquality are in practice around the same size as the ERLGH values, thus one may be utilized as target values for mmol/mol. The findings correspond well with the earlier reports<sup>[43,44]</sup> despite the fact that there are differences in both units and standardization programs between the NGSP/DCCT and IFCC procedures<sup>[35]</sup>. The new target limits of the HbA<sub>1c</sub> results in 2015 for % results are  $\pm 6\%$  and for mmol/mol results  $\pm 8\%$  around the target value and agree well to those reported by Little *et al.*<sup>[17]</sup>, Weykamp *et al.*<sup>[49]</sup> and Lindblad and Nordin<sup>[50]</sup>. In addition, on the basis of CAP surveys, Little *et al.*<sup>[17]</sup> have reported that at the normal HbA<sub>1c</sub> levels (6%-7%) the target value described in % units and with  $\pm 6\%$  around the reference value may be good enough for the diagnosis and follow-up of the treatment of diabetes. Lindblad and Nordin<sup>[50]</sup> reported that when near to the critical level of HbA<sub>1c</sub> of 48 mmol/mol, the maximum allowable difference from the target value should be less than 3.5 mmol/mol, which corresponds to a CV% of 7.3%.

The Uppsala Meeting for Quality Specifications in Clinical Laboratories in early 1990s had one session devoted to the quality of HbA<sub>1c</sub> measurements. The Danish clinical chemists<sup>[51,52]</sup> proposed that a change of



**Table 4** The relationships between the hemoglobin A<sub>1c</sub> units as % values to the mmol/mol values are presented at different levels of hemoglobin A<sub>1c</sub>

HbA <sub>1c</sub>	HbA <sub>1c</sub> (%)	HbA <sub>1c</sub> (mmol/mol)
Reference limits	4.0-6.0	20-42
Diagnosis limit	6.5	48
Treatment limits, adults	7.0	53
children < 6 yr	7.5	69
children 6-12 yr	8.0	64
children 13-19 yr	8.5	58
Poor diabetic balance	9.0	75
Very poor diabetic balance	12.0	108

Equations from the NGSP/DCCT mean values to the IFCC units: % =  $0.095 \times \text{mmol/mol} + 2.15$ ; mmol/mol =  $10.93 \times \% - 23.5$ . The table also shows the most important equations to convert the results described in % units of NGSP/DCCT into the values of IFCC expressed as mmol/mol units<sup>[29]</sup>. HbA<sub>1c</sub>: Hemoglobin A<sub>1c</sub>; NGSP/DCCT: National Glycohemoglobin Standardization Program/the Diabetes Control and Complications Trial Research Group; IFCC: International Federation of Clinical Chemistry.

1.0% from the measured % HbA<sub>1c</sub> value might suggest that the treatment could be necessary whereas a change of 2.0% would demand that treatment should be initiated. This degree of accuracy is necessary that clinicians could feel confident with their decisions to initiate what could well be life-time therapy. These findings are in accordance with the earlier reports<sup>[8,16,20]</sup> and with our present findings. On the other hand, recently in 2015, Weykamp *et al.*<sup>[45]</sup> reported that when using mmol/mol units, the calculated total allowable error could be 4.2 mmol/mol. The HbA<sub>1c</sub> results of current Labquality surveys conducted in 2015, *i.e.*, the mean values of  $50.8 \pm 2.1$  mmol/mol are within this value.

On the other hand, a single laboratory should be able to perform HbA<sub>1c</sub> % measurements with a total analytical variation (within and between series) from between 1.4% to 3.0% when using liquid chromatography<sup>[10,20,21,26]</sup>, this variation due to the analytical procedure is about 4.5 times lower than the measured biological variation of this parameter<sup>[42,53]</sup>. Furthermore, also the differences (errors) between frequent measurements should be as small as possible to ensure reliable follow-up of the treatment of those patients in a stable diabetic state<sup>[16,17,23,31]</sup>. After the proposal from ADA<sup>[18]</sup> to use a diagnostic cutoff limit of HbA<sub>1c</sub> for diabetes, this change demanded that the accuracy of the analytical performance of HbA<sub>1c</sub> had to be better than before the setting of this fixed limit, a fact that has also been criticized.

## REFERENCE VALUES OF HbA<sub>1c</sub>

The commonly used reference intervals for HbA<sub>1c</sub> in % DCCT/NGHP units have been from 4.0% to 6.0%<sup>[16,17,20,49,54]</sup>, the corresponding values for mmol/mol have been from 20 to 42 mmol/mol<sup>[18,50]</sup> as presented in Table 4. The exact values somewhat vary around those

in the table but nonetheless are rather close to those published earlier<sup>[16,17,20,49,50,54]</sup>. In addition, the commonly applied reference values of HbA<sub>1c</sub> in published reports expressed as per cent and mmol/mol are normally stated in the laboratory manuals all around the world as are also the important limits for diagnosis and treatment of diabetes; these manuals also provide the master equations to convert between the system in use and the procedure of IFCC<sup>[23,30-35]</sup>.

## HbA<sub>1c</sub> AND PLASMA GLUCOSE

The commonly used limit of plasma glucose of 7.0 mmol/L<sup>[8,9,17-19]</sup> has been widely accepted when there is a need to diagnose diabetes or to monitor the treatment of the patients. Many physicians treating diabetics like to compare the actual plasma glucose values to the HbA<sub>1c</sub> values and consider that they are assessing some kind of balance of diabetes. However, it was not until 2002 when Rohlfing *et al.*<sup>[55]</sup> collected enough published data to be able to devise an equation describing the well-established ratio between blood mean HbA<sub>1c</sub> and plasma glucose, *i.e.*, (PG/HbA<sub>1c</sub>): Plasma glucose (mmol/L) =  $[1.98 \times \text{HbA}_{1c}(\%)] - 4.29$  ( $n = 1439$ ,  $r = 0.82$ ). However, when inspecting their proposal, it is quite evident that at the same HbA<sub>1c</sub> value, *e.g.*, 8.0%, the plasma glucose concentration may maximally vary from 6 to 15 mmol/L and thus the results are not precise, especially at the higher blood HbA<sub>1c</sub> levels<sup>[49]</sup>. In addition, in subjects with impaired glucose tolerance (plasma glucose value from 5.6 to 6.4 mmol/L or blood HbA<sub>1c</sub> from 5.7 to 6.4 mmol/mol) the 2 h oral glucose tolerance test may be as good or better at revealing both the insulin sensitivity and disturbance in glucose metabolism than can be achieved with a single value of fasting plasma glucose<sup>[56-60]</sup>. These are the reasons why many laboratories do not calculate the mean PG/HbA<sub>1c</sub> ratios but report glucose and HbA<sub>1c</sub> results separately<sup>[41]</sup>. It has also argued that in fulminant (acute) type I diabetes, the specificity of the analysis of HbA<sub>1c</sub> may be doubtful low when compared to the analysis of glycated albumin with a significantly higher specificity<sup>[59]</sup>. This may have a significant role to make a selection of the analyze type in diagnosing of acute diabetes or prediabetes. It is also important that plasma glucose and blood HbA<sub>1c</sub> levels should be assessed in an accredited laboratory if the values are to be used for diagnosis or screening of diabetes<sup>[18,19,23,58-62]</sup>.

## HbA<sub>1c</sub> AND SOME OTHER ASPECTS

Finally, it has been pointed out that in both men and women elevated levels of blood HbA<sub>1c</sub> increase the risk of developing cardiovascular disease<sup>[63]</sup>. In addition, as described in the chapter of analytical problems, many other diseases may affect the HbA<sub>1c</sub> results and cause difficulties for the clinicians. Some diseases other than cardiovascular are also associated with HbA<sub>1c</sub><sup>[62,63]</sup>, *e.g.*, diseases altering iron metabolism.

The point-of care instruments (POC) are being continuously introduced for the analysis of HbA<sub>1c</sub><sup>[25,64]</sup> but there are still many issues associated with their use<sup>[64-66]</sup>: (1) they have not been universally recommended for the diagnosis of diabetes according to the guidelines<sup>[17-19,30]</sup>; (2) many POC users are not participating in the quality control programs of their home country; and (3) the POC analyses are difficult to standardize. In the future, in conjunction with the further development of these methods, especially with the adoption of reliable quality systems, POC analyses may achieve real breakthroughs<sup>[25]</sup>.

## CONCLUSION

This assessment of HbA<sub>1c</sub> analytical procedures and values indicates that a considerable improvement has occurred during the past 30 years with respect to both their the precision and accuracy and these improvements are still on-going as reflected in reduced assay CV% values. The immunoassay techniques have replaced many chromatographic procedures during this time period.

During 2014/2015, the reports from quality assurance systems have confirmed the marked improvement for the quality of HbA<sub>1c</sub> measurements irrespective of whether the results have been expressed in % or mmol/mol units. The acceptance of the mmol/mol system recommended by IFCC for HbA<sub>1c</sub> and the new unit and reference ranges are only becoming slowly accepted outside of Europe where it seems that the parallel reporting for HbA<sub>1c</sub> will continue. The use of a diagnostic cutoff limit for the HbA<sub>1c</sub> value is still not finalized.

The reference analyses from the mean values of the survey results may be used as target values in both % and mmol/mol units assuming that the number of the participating laboratories is high enough to be statistically satisfactory.

The authors also hope that the use of the mmol/mol unit for HbA<sub>1c</sub> can gain worldwide acceptance as this would make it much easier to compare results from different studies and remove the possibility of confusion when units are converted from one form to the other.

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## Methodology in improving antibiotic implementation policies

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### Abstract

The basic requirements of antibiotic prescribing are components of methodology; knowledge, logical reasoning, and analysis. Antimicrobial drugs are valuable but limited resources, different from other drugs and they are among the most commonly prescribed drugs all

over the world. They are the only drugs which do not intentionally affect the patient. They affect the pathogens which invade the host. The emergence and spread of antibiotic-resistant pathogens are accelerated by heavy antibiotic usage. The effective antimicrobial stewardship and infection control program have been shown to limit the emergence of antimicrobial-resistant bacteria. In this respect, education for antibiotic prescribing could be designed by going through the steps of scientific methodology. A defined leadership and a coordinated multidisciplinary approach are necessary for optimizing the indication, selection, dosing, route of administration, and duration of antimicrobial therapy. In scenarios, knowledge is also as important as experience for critical decision making as is designated. In this setting, the prevalence and resistance mechanisms of antimicrobials, and their interactions with other drugs need to be observed. In this respect, infectious disease service should play an important role in improving antimicrobial use by giving advice on the appropriate use of antimicrobial agents, and implementing evidence-based guidelines.

**Key words:** Antibiotics; Infection; Antibiotic resistance; Therapy; Medical informatic applications

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**Core tip:** Treatment of infections has become problematic because of increasing global antimicrobial resistance. One of the major reasons of this is antibiotic misuse and over use. In order to make antibiotic therapy more effective, some guidelines are used. Although guidelines lead to improvements in clinical practice, no guideline can be sufficiently specific that can be applied to all clinical situations. For improving antibiotic implementation strategies, not only consensus-based but also evidence-based scientific methods are needed. This review highlights the knowledge and experimentation of expert physicians under the supervision of antibiotic stewardship.

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## INTRODUCTION

The implementation of antibiotics in cancer chemotherapy and organ transplant patients, make the serious lethal complications of diseases likely treatable. Therefore parallel to medical advances, practice of medicine have changed greatly through antibiotic usage. The early initiation of antibiotics to treat infections reduce morbidity<sup>[1]</sup>. However, 20%-50% of the prescribed antibiotics in hospitals are either unnecessary or inappropriate<sup>[1-3]</sup>. Unnecessary antibiotic using patients are candidates for serious adverse effects even without any effective clinical cure. The misuse of antibiotics also contributes to the growing problem of antibiotic resistance. In the twenty-first century, because antibiotic resistance has become a serious issue for patient safety, improving the use of antibiotics is an important public health problem<sup>[1,4,5]</sup>.

Therefore, antibiotic prescribing should be based on a prudent, well-thought, and rational process. The basic requirement for this is knowledge, logical reasoning and analysis. Meanwhile, the system of rules and procedures for interventions to improve antibiotic use is constantly being updated as scientists and physicians look for new and better ways of making observations, analysis and synthesis<sup>[6]</sup>. For implementation strategies we may need evidence-based methodology. Thus, the below stated steps of a scientific method can be applied for improving antibiotic implementation policies<sup>[7]</sup>: (1) Asking a question; (2) Carrying out background research; (3) Constructing a hypothesis; (4) Testing the hypothesis through an experiment; (5) Analyzing data and drawing a conclusion; and (6) Communicating the results.

## A PRAGMATIC APPROACH TO THE METHODOLOGY OF ANTIBIOTIC IMPLEMENTATION POLICIES

In 2015 Center's for Disease Control and Prevention (CDC) published a report about core elements of hospital antibiotic stewardship programs<sup>[1]</sup>. According to CDC "there is no single template to optimize antibiotic usage". The medical decision is complex and the antibiotic implementation policies should be flexible. Therefore, there is need for defined leadership and coordinated multidisciplinary approach<sup>[1]</sup>. Although the relevant guidelines for antibiotic usage and expert opinions steer and shape the author's vision for judicious antibiotic use in writing this review, the methodology used in this manuscript highlights the author's own ideas.

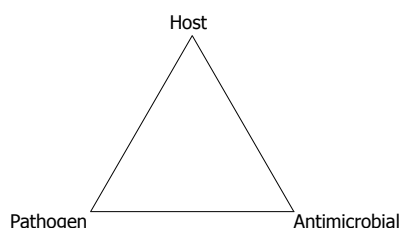
## WHY ARE ANTIMICROBIAL AGENTS DIFFERENT FROM OTHER DRUGS?

Antimicrobial drugs are valuable but limited resources, and they are different from other drugs. Why are antibiotics different from other drugs?<sup>[8]</sup> The three factors which differ antimicrobials from other drugs, compose the important components of antimicrobial therapy<sup>[9]</sup>:

First, antibiotics are among the most prescribed drugs all over the world. Antimicrobials account more than of 30% of hospital pharmacy budgets<sup>[4]</sup>. In developing countries one third of the budget reserved for health care is also spent for antibiotics<sup>[10]</sup>. Therefore the cost effectiveness of antimicrobial treatment of bacterial infections is important<sup>[11]</sup>. The excessive consumption and prescription of antibiotics ended up with high costs; so the "the Ministry of Health in Turkey" restricted the prescription of excessively used expensive antibiotics nationwide<sup>[10]</sup>. With this new policy the responsibility of the prescription of antimicrobials is given to the infectious disease (ID) specialist physicians. Nevertheless, the comparative cost of antibiotics is only one of the factors in determining the physicians' antibiotic choice, and it should never be the most important factor<sup>[11]</sup>.

Second, antimicrobials are the only drugs which do not intentionally affect the patient; this feature differs antibiotics from other drugs. Antibiotics can affect both the pathogen and the colonizing flora<sup>[8,9]</sup>. Antimicrobial therapy is based on the characteristics of a patient and a drug, as well on the characteristics of the microorganisms<sup>[8]</sup>. This complex relationship between the patient, antimicrobial, and microorganism is defined as the cornerstones of a triangle<sup>[8]</sup>, as shown in Figure 1. In this concept host presents the antimicrobial agent to the pathogen<sup>[9,12]</sup>.

The selection of an antibiotic for the appropriate empirical antimicrobial therapy has become increasingly difficult. Physician has to know many different aspects of IDs; such as immunological and genetic host factors, microbial virulence, pharmacokinetics (PKs) and pharmacodynamics (PDs) of drugs<sup>[8]</sup>. PKs describes the action of drugs in tissues and body fluids over a period of time, including the processes of absorption, distribution, and excretion<sup>[13]</sup>. PDs studies the relationship between the biochemical and physiological interactions of drugs on the body or on microorganisms. Physiological effects are associated with primary and underlying disorders, aging, interactions with other drugs, etc. Meanwhile, the time course and the concentration of the therapeutic agent at the infection site, and its adverse effects have to be concerned<sup>[13]</sup>. PDs, with PKs, explains the relationship between the dose and response. The postantibiotic and bactericidal action of antimicrobials related to drug's PD effects, have to be known for optimal dose regimens. In addition, the optimal dose and dosing intervals have also to be determined according to the minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations of the antimicrobials against microorganism groups<sup>[13]</sup>.



**Figure 1** The relationship between the patient, antimicrobial and microorganism.

Some drugs, such as the  $\beta$ -lactams, exert their maximal effect when antibiotic load exceed the MIC of the microorganism for a certain period of time between doses. Such agents are known as “time-dependent” antibiotics and they are also recognized with the PD indices as “time above MIC”<sup>[4,14]</sup>. For time-dependent antibiotics during the dosing interval, the serum drug concentrations should achieve at least 40% to 50% of the MIC of the causative pathogen<sup>[13]</sup>. These studies support the concept of administering  $\beta$ -lactam antibiotics at shorter intervals, prolonging the infusion time, or even administering by continuous infusion for serious systemic infections<sup>[4,14]</sup>. Beta-lactam antibiotics show an inoculum effect. When the bacterial density is low, the concentration of the required  $\beta$ -lactam could be low, and vice versa<sup>[13]</sup>. The aminoglycosides and fluoroquinolones, on the other hand, exhibit “concentration-dependent” killing. For these and similar agents, the PD indices are determined as “peak/MIC” (peak serum concentrations divided by the MIC) and as “24-h area under curve/MIC”. These ratios show that giving the fluoroquinolones and aminoglycosides by once daily dose are encouraging from a PD point of view<sup>[4,14]</sup>. Thus the high peak levels obtained after short infusion dosing or high exposures during 24 h, cause the most rapid killing of the infecting pathogen<sup>[4,14]</sup>. In most clinical settings, administering aminoglycosides as a total single daily dose are recommended and have become the standard of application. On the other hand, the glycopeptides exhibit time-dependent killing. They also show slow bactericidal activity and a short post-antibiotic effect *in vitro*<sup>[13]</sup>. Studies of PK and PD account for the basis of dose optimization<sup>[4]</sup>.

Third, the overuse of antibiotics in human health care and animal feeds, the increased use of invasive devices and procedures, and invalid infection control practices have resulted in antibiotic resistance. The resistance is acquired by mutational change or by transfer and acquisition of resistance-encoding genetic material in health care settings<sup>[15]</sup>. So, multidrug resistant bacteria is the major cause of failure of the treatment of IDs which the clinician has to deal with<sup>[15]</sup>. The overuse of other drugs does not introduce any negative effect to other medications as antimicrobials do. Therefore, to limit the transmission of emerging multidrug-resistant organisms, implementation of regional antibiotic usage data has to be developed<sup>[5]</sup>.

It is known that changing the attitude of the professional who is prescribing an antibiotic, is not easy. So, to aide the physicians attitude for antibiotic usage, early antimicrobial stewardship interventions have been practiced at the postgraduate level<sup>[3,16]</sup>. The IDs Society of America (IDSA) has defined the antimicrobial stewardship. With this, IDSA stated that for maximum clinical cure, the indication, selection, dosing, route of administration and duration of antimicrobial therapy have to be optimized. In addition while selecting antimicrobials, it should be taken into account that they will produce no damage; including toxicity, selection of pathogenic organisms, such as *Clostridium difficile*, and emergence of resistance<sup>[4,5,8,16]</sup>. To avoid resistance selecting pressure, the antibiotics have to be given for the shortest possible duration, in adequate doses, and with the least broad-spectrum<sup>[8]</sup>.

The systematic ways of doing, teaching, and studying antibiotic implementation strategies constitute the main issue of this manuscript. In other words the question of what could be the system of methods and principles used in improving antibiotic implementation practices, should be answered. James Bach states that “there is no consensus about what practices are the best, unless consensus means; people I respect also say they like it. There are practices that are more likely to be considered good and useful than others, within a certain community”. But... so what? “Good practice is not a matter of popularity. It’s a matter of skill and context” (James Bach; founder and operator of exploratory software company Satisfice, Inc.).

## INFECTIONS AND ANTI-INFECTIVE THERAPY

The IDSA and the Society for Healthcare Epidemiology of America (SHEA) published Guidelines for Antimicrobial Stewardship Programs in 2007<sup>[4]</sup>. These guidelines do not pose as a substitute for clinical judgement<sup>[4,16]</sup>. In selecting the appropriate antimicrobial agent for therapy of an infection, some important factors should be kept in mind<sup>[14]</sup>. First, the identification and the source of infecting organism must be known or, at the very least, it must be possible to arrive at a statistically reasonable guess as to its identity on the basis of clinical information<sup>[14]</sup>. Also, the environment where the infecting organism is contracted (community or hospital) should be questioned. Second, information about the susceptibility of the infecting organism, or likely susceptibility, must be as accurate as possible<sup>[14]</sup>. Finally, a series of factors specific to the patient who is being treated must be considered to arrive at the optimal choice of antimicrobial agent<sup>[14]</sup>.

Today most antibiotic treatment is empirical. In 2012 as stated by Livermore<sup>[17]</sup>, “in general most diagnostic microbiology laboratory practice moves at the speed it did in Fleming’s time: 1 d from specimen to culture and another from culture to identification and susceptibility

data". In practice, for critically ill patient empirical therapy is started as early as possible when evidence of infection is observed<sup>[18]</sup>. The antibiotic guidelines combined with the physician's experience shape the therapeutic choices for empirical antimicrobial usage<sup>[18]</sup>. Meanwhile, several methods for the rapid identification of pathogenic bacteria are available in clinical specimens. A Gram stain-preparation is perhaps the simplest and least expensive method to determine the presence of bacterial and some fungal pathogens. Final and definitive identification of pathogenic organisms still often require a culture technique. It is extremely important that appropriate specimens be obtained for culture before beginning antimicrobial therapy, to test the susceptibility of agents that may be used in therapy<sup>[14]</sup>. Once anti-infective therapy starts, cultures often become sterile, even though viable organisms remain in the host. So samples taken before starting antimicrobial therapy is important for the identification and antimicrobial susceptibility of the organisms causing an infection<sup>[14]</sup>. If fast usage of molecular microbiology techniques to identify pathogens and fast detection of their resistances in primary clinical specimens could be achieved, the individual patient may benefit from early use of the most powerful antimicrobial<sup>[17]</sup>.

For optimal therapy, a number of host factors that may influence the efficacy and toxicity of antimicrobial agents should be considered. The age of the patient, the presence of genetic and metabolic abnormalities, pregnancy, renal and hepatic function all have significant effect on the toxicity of a given antimicrobial agent<sup>[14,19,20]</sup>. Another consideration in selection of an appropriate antimicrobial is the site of infection. The concentration of the antibiotic at the site of infection should be  $\geq$  the MIC of the infecting organism for the antimicrobial therapy to be effective<sup>[14,19,20]</sup>.

For judgement of the effectiveness of therapy, clinical improvement is the best directory; although it is not always easy to observe objectively<sup>[21]</sup>. As stated by Paterson *et al*<sup>[21]</sup>, "culture results are sometimes negative, yet an infection is clinically present, or culture results are positive, but indicate the presence of colonization rather than true infection". In such situations, switching an antimicrobial agent has to be questioned in detail; "Don't change a winning team"<sup>[21]</sup>. Besides, for serious infections such as endocarditis long-term therapy is needed, so for monitoring clinical improvement physician has to observe the patient for a longer period of time. Another point is that fever should not always be the only factor to judge the success of antimicrobial therapy. Although a febrile patient under antimicrobial therapy could be related to treatment failure, there are also other factors which have to be considered; such as, drug fever, abscess formation, or other infection with an opportunistic pathogen<sup>[14]</sup>. Some surgical interventions in such occasions, like abscess formation drainage, could be as important as anti-infective therapy and should be considered by the ID specialist and by the surgeon<sup>[19]</sup>.

## ANTIMICROBIAL STEWARDSHIP PROGRAM

Antibiotic implementation policies should be flexible<sup>[1]</sup>. Meanwhile, the success depends on a defined leadership and a coordinated multidisciplinary approach<sup>[1]</sup>. For optimum decision making in prescribing antibiotics, physicians have to have sufficient knowledge of IDs, infecting pathogens, and antimicrobials. So the director of the program should be ID specialist, or ID specialist should co-direct the program with a clinical pharmacist. Thus, the key members of an antimicrobial stewardship team is constituted<sup>[4,5]</sup>. For antimicrobial resistance surveillance a clinical microbiologist, and for the computer support an information system specialist are also needed<sup>[4,5]</sup>. The IDs physician or a clinical pharmacist with IDs training has to have interactions with the prescriber physicians. They should perform a prospective audit and a feedback system, serving to reduce the inappropriate use of antimicrobials<sup>[4,5]</sup>.

What could be done for to avoid antimicrobial resistance? Many investigators in nowadays try to figure out inappropriate antibiotic use and they use this figure as a surrogate marker<sup>[4,5]</sup>. For effective antimicrobial stewardship many interventions are recommended such as; formulary restriction and preauthorization, antimicrobial order forms, antimicrobial cycling<sup>[4,5]</sup>. However, the formulary restriction should be dynamic. The recommendation rules of the routine uses of these basic implementation procedures are not based on sufficient data. Therefore, to increase the acceptance of stewardship strategies, continuous education programs have to be considered<sup>[4,5,8]</sup>. In addition, local microbiology laboratories' antibiotic sensitivity results and resistance patterns, and infection control programs should be taken into account to improve antimicrobial utilization<sup>[4,5]</sup>.

### ***Interaction between inappropriate antibiotic use and antimicrobial resistance***

Although, antibiotic resistance is caused by mutations in bacteria's genes, inappropriate use of antibiotics accelerates the emergence of antibiotic-resistant bacteria<sup>[15]</sup>. However, antibiotics kill susceptible bacteria but resistant bacteria can overgo to grow and multiply. The people who have not taken antibiotics are also under risk of getting infected with antibiotic resistant bacteria strains. In this respect, there seems to be a complex relationship between antibiotic use and antimicrobial resistance<sup>[15,22]</sup>. The relationship is affected by antimicrobial PDs, mutant selection windows (MSWs), the mutational or acquired resistance<sup>[15,22]</sup>.

The MSW is a novel *in vitro* concept. It is defined as "the zone between MIC and mutant prevention concentration"<sup>[23]</sup>. In other words, it means "the ability of antibiotics to prevent the emergence of mutant"<sup>[23]</sup>. Because antibiotic susceptibilities of different bacteria strains may differ, they might have different selective



windows, depending on the MIC of a given antibiotic. When resistance to antibiotics has reached high levels, it is difficult to prevent new mutants<sup>[24]</sup>.

Another interaction between antibiotic usage and antimicrobial resistance can be MIC creep<sup>[25]</sup>. To explain this phenomenon for at least last 20 years, vancomycin has been used as the cornerstone for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA)<sup>[25]</sup>. In healthcare settings, very small percent of *S. aureus* isolates (< 1%) select resistance to vancomycin. There are also very rare vancomycin-resistant *S. aureus* and vancomycin intermediate-resistant *S. aureus* strains. "Vancomycin MIC creep" is the rising of vancomycin MICs among vancomycin susceptible *S. aureus* (vancomycin MIC  $\geq 2$   $\mu\text{g/mL}$ ). Vancomycin MIC creep is seen in patients with recent vancomycin use. This situation causes difficulty in the treatment of complicated staphylococcal infections like bacteremia, pneumonia, etc<sup>[25]</sup>. Various poor clinical outcomes and increased costs of hospitalization have shown to be associated with infections caused by *S. aureus* isolates with higher vancomycin MICs<sup>[25]</sup>.

Although, for successful empirical therapy broad-spectrum coverage is necessary, excessive aggressive therapy may cause the emergence of antibiotic resistance<sup>[21]</sup>. After getting antibiotic sensitivity results, the spectrum of therapy should be narrowed. The value of such streamlining and de-escalation therapy, have to be evaluated fully by the IDs physicians<sup>[21]</sup>.

### Formats of educational curricula

For antibiotic prescribing behavior of hospital programs, education is an essential element<sup>[8]</sup>. According to Pulcini *et al*<sup>[8]</sup> "targeted antibiotic sessions in the format of problem-based learning are absolutely necessary. It is important to identify the topics or concepts that benefit from a disease- (e.g., acute bronchitis) or problem- (e.g., antimicrobial resistance) oriented, rather than a pathogen- (e.g., MRSA) oriented, or a drug- (e.g., antibiotic classes) oriented approach". In the United Kingdom, for the same purpose a website is performed as "the Prudent Antibiotic User" (PAUSE, [www.pause-online.org.uk](http://www.pause-online.org.uk)). It is aimed to be used in the undergraduate medical curriculum. PAUSE has provided standardized teaching aides for all educators of antibiotic prescribing that have based on cases<sup>[8]</sup>. Meanwhile, "the European Society of Clinical Microbiology and IDs study group for Antibiotic Policies" (available at [www.esamid.org/esgap](http://www.esamid.org/esgap)) has performed postgraduate education courses; "Antimicrobial Stewardship: Measuring, auditing and improving", held bi-annually before "the European Conference on Clinical Microbiology and IDs", internationally<sup>[8]</sup>. It is pointed out that antimicrobial stewardship programs have mainly been implemented at the postgraduate level. Instead, it seems antimicrobial stewardship is likely to be more successful if started earlier with undergraduate programs, "at the time when knowledge, attitude, and behavior of professionals are

being shaped" as stated by Pulcini *et al*<sup>[8]</sup>.

For improving antibiotic usage educational programs should be based on the needs of the hospitals<sup>[1]</sup>. The effective element in one hospital may not be effective in another<sup>[8]</sup>. To optimize antibiotic selection and duration, there are written national guidelines. These guidelines cover systemic infections such as respiratory tract, urinary tract, intra-abdominal, skin and soft tissue infections, and as well, documentations for surgical prophylaxis<sup>[1]</sup>. The Cleveland Clinic published a guideline booklet (2012-2013) for antimicrobial usage<sup>[26]</sup>. The information given in the introduction section of that booklet was striking; in that the authors underlined that the materials were subject to change and appropriate medical judgements were necessary relative to individual patient's needs<sup>[5,26]</sup>. Meanwhile to improve antibiotic usage, Fishman in CDC's safe Healthcare Blog proposed some simple steps that could be overviewed for antimicrobial therapy<sup>[16]</sup>: (1) "Never treat viral syndromes such as acute bronchitis with antibiotics, even when patients demand therapy; (2) Use fluoroquinolones cautiously. Not only fluoroquinolone resistance is rising at an alarming rate, but this group of drugs causes resistance to many other antibiotics and is associated with the new more virulent strain of *C. difficile*; (3) Antibiotics used for surgical prophylaxis should rarely be given for more than 24 h; post-operative doses are not required in many cases; (4) Refine your antibiotic choice once culture data is available and always use the drug with the narrowest spectrum; and (5) Double coverage is rarely necessary once antimicrobial susceptibilities are known".

Empirical therapy is being started at the first visit of the patient. Soon after the hospitalization of the patient some additional laboratory data are also available. Bacterial culture and susceptibility test results are achieved in 48 h. So, all clinicians should review their initial antibiotic choices after sufficient laboratory data are in hand<sup>[1]</sup>. According to CDC (2015) for the management of antimicrobial therapy, below key questions have to be answered<sup>[1]</sup>: (1) "Does this patient have an infection that will respond to antibiotics? (2) If so, is the patient on the right antibiotic(s), dose, and route of administration? (3) How long should the patient receive the antibiotic(s)? and (4) Can a more targeted antibiotic be used to treat the infection (de-escalate)?"

At the beginning of therapy, broad-spectrum antibiotics which are effective to the most likely pathogens are started. The de-escalation means; knowing and collecting bacterial culture results of the organisms for later microbiological judgement while targeting streamlining to a more narrow-spectrum antimicrobial regimen 2-3 d later. This intervention is performed by the means of clinical status and culture results of the patient<sup>[27]</sup>. Thus, by definite or pre-emptive approaches to the pathogen, a decrease in antimicrobial resistance pressure and antibiotic costs could be achieved by de-escalation strategies<sup>[27]</sup>.

### **Guidelines for antimicrobial usage**

The guidelines for antimicrobial usage aim to encourage prudent prescribing of antimicrobials for empirical treatment of infections, and thereby improve the results of treatment, reduce drug related toxicities, and limit the emergence of resistant strains<sup>[28,29]</sup>. They contain advice on the appropriate initial management of common conditions and include route of administration, dose, and duration of treatment. Where a dose range is recommended, the patient's status should be considered to define the need for using higher doses in more severe infections and considering lower doses for certain patients' groups such as the elderly, those with a low body mass index, and those with liver and kidney failures<sup>[28,29]</sup>. Recommendations for antibiotics in medical prophylaxis are also given. Surgical prophylaxis documents are available separately<sup>[28,29]</sup>.

### **Essentials of antimicrobial use guidelines**

**Dose optimization:** Optimal dosing of antimicrobials depends on patient's age, renal function, weight, etc., and causative organism. Site of infections (endocarditis, meningitis, osteomyelitis, etc.), and PK and PD parameters are also have to be taken in account.

Continuous infusion of  $\beta$ -lactams, once-daily dosing of aminoglycosides, and increased dosing of fluoroquinolones for *Streptococcus pneumoniae* and for *Pseudomonas aeruginosa* in nosocomial pneumonia are good examples depending on PK and PD parameters<sup>[4,30]</sup>.

**Parenteral to oral conversion:** To reduce the length of hospitalization and to lower the costs of antibiotics, switching from intravenous (IV) to oral (PO) therapy is recommended when patients become stable. Generally IV forms of antimicrobials are more bioavailable and have greater effects, meanwhile some oral forms of antibiotics provide high serum levels as comparable as parenteral forms<sup>[31,32]</sup>. Some antibiotics don't have an oral form, in such situations an oral antibiotic from a different class with similar spectrum of activity is advised<sup>[33]</sup>.

**Combination therapy:** Combination therapy is unnecessary when the pathogen and the susceptibility are known. Combination therapy is recommended for the prevention of resistance. When an organism load is heavy and when there is a high probability of mutational resistance during therapy, combination of antimicrobials are to be considered. Good examples which are supporting the combined anti-infective therapy are tuberculosis or HIV infection<sup>[4]</sup>. Other indication of combined antibiotic therapy is enterococcal endocarditis. Combination drug therapy in some situations may prevent or delay the emergence of resistance during exposures within the MSW<sup>[14]</sup>.

Rifampin has good CSF penetration but selects resistant mutants when used alone, so its combined use with other antimicrobials is recommended. Rifampin with vancomycin combination in staphylococcal CSF

shunt infections is accepted as the best choice<sup>[34]</sup>.

Besides, some medications penetrate tissues better than others. For example, if the primary focus of *Pseudomonas* meningitis treated with meropenem is mastoiditis, it is advised to combine meropenem with an aminoglycoside antibiotic which penetrates to the skeletal system better.

**Microbiology laboratory:** For the identification of microorganisms and for the determination of bacterial susceptibilities, the clinical microbiology laboratory plays an important role. For qualitative susceptibility testing principles, national and international guidelines are used. Using disk-diffusion (Kirby-Bauer) techniques by measuring zone diameters, or by calculating MICs, ESBLs can be detected. For inducible clindamycin resistance of *S. aureus* D test is also performed with disk-diffusion method<sup>[4]</sup>. The detection of such parameters regarding resistance leads to the selection of appropriate antimicrobials.

**Duration of therapy:** To control the bacterial infection and prevent relapse, the duration of antibiotic therapy is to be sufficient. When optimizing therapy for an infection, one should consider the person's immune status, the infecting agent and the focus of infection. In most clinical scenarios, because the recommended duration of therapy depending on the clinical situation is flexible, it is usually based on expert opinions<sup>[35]</sup>. To base the clinical decision on laboratory data, some biomarkers such as C-reactive protein or procalcitonin (PCT) have been increasingly studied<sup>[35]</sup>. Today, PCT is the only biomarker which has been generally accepted to help the decision-making in discontinuing antibiotic therapy in sepsis and pneumonia of adults. In clinical practice the advised use of PCT levels are the 1<sup>st</sup> day, 2<sup>nd</sup> or 3<sup>rd</sup> days, and every 48 h of antibiotic commencement until antibiotic discontinuation<sup>[36]</sup>. Meanwhile, the use of procalcitonin levels has not been excessively studied in all infection types. Besides, the high cost in determining procalcitonin level can limit its use<sup>[35]</sup>.

Longer antibiotic therapy encourages the development or acquisition of antibiotic-resistant organisms. Ventilator-associated pneumonia has traditionally been treated for a long course (14-21-d). However, prolonged antibiotic therapy has caused the emergence of multidrug-resistant strains. It is also associated with high toxicity and high costs. In patient groups who are receiving antibiotics for shorter periods of time (8 d) antibiotic resistance is less common. The studies support that if the choice for initial antibiotic therapy is prudent, 8-d duration of therapy for ventilator-associated pneumonia may be appropriate, and the clinical course seems to be favorable after stopping antibiotics<sup>[37]</sup>.

### **Emerging developments in antibiotic stewardship**

In antibiotic stewardship programs the commonly used diagnostic test is PCT. There are also novel tests which are not used frequently, such as fluorescence *in situ*

hybridization and matrix-assisted laser desorption/ionization time of flight mass spectrometric analysis<sup>[1]</sup>.

Recently, measuring antibiotic use as either days of therapy, or defined daily dose (DDD) is becoming favorable. With the use of DDD hospitals may have the opportunity to compare their antimicrobial consumptions with that of other similar hospitals<sup>[1,4]</sup>.

## DECISION MAKING IN CLINICAL PRACTICE

### Study (clinical practice)<sup>[2]</sup>

The Center of Disease Control (CDC) evaluated hospitalized patients with the aim of improving inpatient antibiotic prescribing. They used a national administrative database, "MarketScan Hospital Drug Database", and CDC's "Emerging Infections Program" for the study<sup>[2]</sup>. First, judicious antibiotic usage was described. Second, the principles of improving antibiotic prescribing data were illustrated in selected clinical cases. Third, when antibiotic usage had been improved, the decrease in *Clostridium difficile* infections were estimated. The results of the study had been reported to the "National Healthcare Safety Network" with the findings of using selected antibiotics in variable lengths of time<sup>[2]</sup>.

### Scenario (decision making-1)<sup>[38]</sup>

An 83-year-old man with fever (38.7 °C), dysuria, and chills is admitted to the ward. The patient's medical history and clinical examination findings are recorded. On the basis of symptoms and signs of the patient, the urine examination is found to be necessary. After observing urine microscopy, the physician has to decide starting IV antibiotic treatment with the diagnosis of severe urinary tract infection<sup>[38]</sup>.

The most common pathogens of urinary tract infection are reviewed as *Escherichia coli* (*E. coli*) (60%), *Proteus mirabilis* (10%), and *Klebsiella pneumoniae* (10%) in people of that age. It is known that the microbiology laboratory of the hospital reports the susceptibility of common pathogens yearly. From those data the physician can estimate that the sensitivity of the mostly used antibiotics will be as follows: Imipenem 100% third generation cephalosporins 95%, gentamicin 92%, second generation cephalosporins 75%, and ampicillin 40%<sup>[38]</sup>. The clinician wants to choose the antibiotic which is supposed to be matching with the *in vitro* susceptibility of the pathogen, to give the patient the best chance of recovery. However, you have been told by the head of the administration that third generation cephalosporins and imipenem are expensive, and they should rather not be the first choice if patient's clinical status is affordable<sup>[38]</sup>. With gentamicin treatment nephrotoxicity and ototoxicity develop in the ratio of 10%. In addition, there can be more severe side effects, and because of the patient's advanced age, the risk of nephrotoxicity can be high. On the other hand, the *in vitro* susceptibility pattern of the pathogen will be

announced within 48 h, so that the clinician can change the antibiotic with the susceptible one<sup>[38]</sup>. According to Leibovici *et al*<sup>[38]</sup>, "the thought that a healthy and active 83-year-old man will need hemodialysis because of a drug that you have prescribed is frightening; so the physician decide to order a second generation cephalosporin. Still, the clinician are not altogether satisfied with the fact that he has reduced the antibiotic coverage by 7%-15% because of his concern about the high costs and fear of side effects associated with other antibiotics<sup>[38]</sup>. He wishes that the balance of benefits and detriments of antibiotic drugs could have been weighed at leisure somewhere else and that you had evidence based guidelines to help him make a choice"<sup>[38]</sup>.

Two days after admission, the *E. coli* isolate identified from urine sample of the patient is informed as susceptible to second generation cephalosporins and the patient is doing well as expected with the result. "But still the physician wonders whether prescribing a drug that affords less than the maximum coverage is the right thing to do", as Leibovici *et al*<sup>[38]</sup> emphasize. The authors also highlight that, "this may well slow down the development of resistance and give future patients (to whom you have a duty too) a better chance for an uneventful recovery but your main duty is to your present patient<sup>[38]</sup>. How do you balance the two duties? The important decision in antibiotic treatment turns out to be a choice between present and future patients"<sup>[38]</sup>.

As antibiotic resistance increases, choosing prudent antibiotics for the empirical treatment of serious infections become even more difficult<sup>[39]</sup>. As stated by Retamar *et al*<sup>[39]</sup> "in such situations, physicians face a dilemma: To provide a very-broad-spectrum empirical coverage, accepting that on many occasions it will be excessive and might contribute to further resistance selection, or to use a narrower-spectrum empirical regimen, accepting that it may not cover the causative pathogen and might require correction once the susceptibility results are known".

### Scenario (decision making-2)

A 52-year-old male was admitted to the emergency room at the fifth day of right inguinal hernia operation. He had 39.2 °C fever, erythematous rash on the right lower extremity and systemic signs of toxicity (tachycardia and hypotension). The surgeon learned that the patient became erythematous at the site of the surgical wound the third day. He examined the patient; opened the incision and observed the wound as deceptively benign in appearance and obtained culture specimen from the wound. The ID specialist diagnosed the patient as septic shock and moved the patient to the intensive care unit (Özgenç O; unpublished data).

In patients with clean extra-abdominal operations, surgical site infections (SSIs) are generally seen in the ratio of 2%-5%. This ratio can reach up to 20% in abdominal surgery<sup>[28]</sup>. Surgical complications associated with infection at the site of incision generally are not

visible till two weeks. Some small portion can be apparent in five days after the operation. Late infections are less probable but 30 d period of following-up of infectious complications are recommended<sup>[40]</sup>.

In this situation, how could the surgeon evaluate the patient? (1) Not all postoperative fevers are related to SSIs and there could also be non-infectious erythematous signs around the surgical incision; (2) Soft-tissue infection within the 48-72 h after an operation is not common and an infection can rarely cause fever; and (3) In this respect, could the surgeon exclude the possibility of SSI for this case? The answer is no. After two days the microbiology laboratory reported that *S. aureus* has grown from the culture of the above mentioned patient's wound specimen.

Early post-SSIs are due to *Streptococcus pyogenes* or *Clostridium* species<sup>[40]</sup>. When high fever is measured or systemic signs of infection are observed in few days after surgery, direct examination of the wound is necessary<sup>[40]</sup>. Toxic shock syndrome is another rare cause of early SSI due to staphylococci<sup>[40]</sup>. In such cases the wound misleads to benign appearance, although there is serious infection beneath the incision site. Erythroderma may be seen early in the course of the infection. Fever, hypotension, hepatic and renal impairment, and sometimes diarrhea may also be the early findings. Before beginning antistaphylococcal therapy, the incision should be opened and specimen for culture should be obtained<sup>[40]</sup>.

This presented scenario highlights that knowledge is as important as experience. The physician has to know all the clinical aspects of surgical manipulations which may lead to early or late SSIs, or to some other complications. "The devil is hidden in the individual details" as stated by Fishman<sup>[41]</sup>. These hidden details could be lightened by the experienced and well-informed ID specialists.

## OVERWHELMING ANTIBIOTIC RESISTANCE - KNOWLEDGE AND EXPERIMENTATION

"The Antibiotic Resistance Monitoring and Reference Laboratory" have shown that during the last decades, resistance to antibiotics have shifted from Gram-positive to Gram-negative bacteria. With the development of new antimicrobials and with strict infection control policies, resistance to MRSA has fell down. New antibiotics; daptomycin, linezolid, and tigecycline, active against MRSA have been discovered<sup>[42]</sup>. After the development of conjugate vaccine, the resistance problem to pneumococci has seemed to be declined. The problem with enterococci is greater. In serious manifestations with enterococci such as in endocarditis, high-level aminoglycoside resistance is great concern since bactericidal activity is needed. In this situation, the clinician refers to daptomycin's bactericidal effect with its high activity<sup>[42]</sup>.

With Gram-negative pathogens the resistance rate is twice that of Gram-positive bacteria<sup>[17]</sup>. The spread of extended spectrum  $\beta$ -lactamases (ESBLs) in Enterobacteriaceae have resulted cephalosporin resistance to go up<sup>[43]</sup>. In severe infections with ESBL-positive bacteria cephalosporins have not shown good clinical outcomes. Moreover, in the last decade there have risen a problem with the striking emerging of CTX-M ESBLs. Although TEM and SHV ESBLs have associated with *Klebsiella* spp., CTX-M types confine to *E. coli*. The CTX-M ESBLs have both spread amongst hospital and community patients. They are mostly isolated from elderly patients and from patients who have hospital contacts<sup>[17]</sup>.

The most CTX-M positive *E. coli* is resistant not only to cephalosporins, but also to quinolones and trimethoprim-sulfamethoxazole which are commonly used antimicrobials for the treatment of urinary tract infections<sup>[44]</sup>. Instead, susceptibility rates indicate that fosfomycin and nitrofurantoin can be considered as important oral treatment options for the treatment of uncomplicated urinary tract infections<sup>[44]</sup>. By the way, there is concern about the rise of rapid and disturbing spread of ESBLs, Amp C enzymes, and quinolone resistance against Enterobacteriaceae. Carbapenem resistance, caused by *Klebsiella pneumoniae* carbapenemases, metallo- $\beta$ -lactamases, and OXA-48 is decreasing reliance on carbapenems<sup>[17,42]</sup>. Last, the gonococcus is also developing resistance to most antibiotics (fluoroquinolones and cefixime) which can easily be used orally<sup>[17]</sup>.

In the last decades third-generation cephalosporins and fluoroquinolones were used alone for urinary infections in many hospitals. This phenomenon has also resulted in selection of resistant mutants such as *C. difficile*<sup>[17]</sup>. Thus, the use of  $\beta$ -lactamase inhibitor combinations has been increased; principally piperacillin/tazobactam and amoxicillin/clavulanic acid 3-4 fold<sup>[17]</sup>. Piperacillin/tazobactam shows less selective pressure than cephalosporins for *C. difficile*, and also produces less selective pressure for vancomycin-resistant enterococci, and for some ESBL-producers, than cephalosporins do<sup>[17]</sup>.

On the other hand, another important antimicrobial resistance mechanism is the expression of chromosomally encoded multidrug efflux pumps<sup>[45]</sup>. During fluoroquinolone monotherapy of *P. aeruginosa* infections, these efflux producing mutants can be selected and especially if the given drug dosages are not adequate, fluoroquinolones can select multidrug-resistant organisms. Therefore, in the treatment of serious infections among hospital patients, the usage of fluoroquinolones can be limited<sup>[46]</sup>.

For the treatment of serious infections by ESBL-producer microorganisms, carbapenems remain as almost only active drug choice<sup>[42]</sup>. Because of the fact that cephalosporin resistance in Enterobacteriaceae has risen, carbapenem use has increased<sup>[43]</sup>. This increased use of carbapenems has resulted in carbapenemase



enzymes selection which are much more commoner in non-fermenters than in the Enterobacteriaceae. The carbapenemases are mostly produced by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, due to OXA carbapenemases and to chromosomal mutations in the non-fermenters, respectively<sup>[42]</sup>. Meanwhile, against carbapenemase-producers, colistin, tigecycline, and fosfomycin are the treatment options with some adverse effects such as toxicity and resistance<sup>[17]</sup>.

To delay antibiotic resistance, heterogenous use of different antimicrobial classes within a period, can be preferred. Prescribing antibiotics from different groups in equal proportions are shown to be effective for preventing the dissemination of multi-drug resistant pathogens<sup>[47,48]</sup>.

Antibiotic resistance has become a major public health priority<sup>[42]</sup>. In this respect, Australian Government has announced "the first National Antimicrobial Resistance Strategy for the years of 2015-2019", for "antibiotic misuse and resistance" (AMR)<sup>[49]</sup>. Because the prevalence of AMR is increasing all over the world, there is a great concern that we will be out of treatable antibiotics. The World Health Organization and other authorities are aware of the fact that pharmaceutical industry is far beyond the increasing resistance pace of microorganisms for to develop new anti-infective drugs against them<sup>[49]</sup>. So, the physicians should achieve wide knowledge and experience regarding multidrug-resistant bacteria, to prevent the failure of treatment of IDs both in the hospital and in the community.

## IMPORTANCE OF ANTIMICROBIAL SURVEILLANCE PROGRAMS

Antimicrobial surveillance programs (SENTRY, MYSTIC, European Antimicrobial Resistance Surveillance System, etc.) have become necessary for to control antimicrobial resistance and to guide appropriate clinical decisions for anti-infective therapy<sup>[50]</sup>. From antimicrobial surveillance studies there can be obtained many information serving for antimicrobial resistance data. These programs detect emerging and changing resistance problems and patterns, as well guide appropriate antimicrobial therapy, based on antibiotic susceptibility data. These findings and results will help to the progress of the development of new antibiotic compounds<sup>[50]</sup>.

## CONCLUSION

In summary, selection of the appropriate antimicrobial depends on understanding of the likely pathogens and local susceptibility patterns. In choosing the right antibiotics, the properties of the antimicrobials; such as PK and PD profiles, activity and potency, tolerability and safety, are all important factors<sup>[51]</sup>. For the treatment of serious infections, the prudent antibiotic with the narrowest spectrum should be started early with the right dose, and should be given for an adequate

duration. These antibiotic implementation principles early in the ID course, play the key role in initial appropriate antibiotic prescribing<sup>[19,51]</sup>. By the way, initial appropriate antibiotic treatment has been shown to reduce mortality, length of stay in intensive care unit and hospital. Early correct antibiotic choices have also served to the reduction in antimicrobial costs<sup>[19,51]</sup>. In this respect, it is underlined that ID service being an expert in the field, plays an important role in improving antimicrobial usage, by giving advice on the judicious use of antimicrobial agents and by developing evidence-based guidelines under the light of antibiotic implementation policies<sup>[50,52]</sup>. The ID specialist in his tough way can adopt antibiotic resistance as a "chronic disease", instead of trying to overwhelm it<sup>[53]</sup>.

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Basic Study

## Rat model of cholelithiasis with human gallstones implanted in cholestasis-induced virtual gallbladder

Marlein Miranda Cona, Yewei Liu, Ting Yin, Yuanbo Feng, Feng Chen, Stefaan Mulier, Yue Li, Jian Zhang, Raymond Oyen, Yicheng Ni

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### Abstract

**AIM:** To facilitate translational research on cholelithiasis, we have developed a rat model of human gallstones by exploiting the unique biliopancreatic features of this species.

**METHODS:** Under anesthesia, 16 adult rats of equal genders underwent two times of abdominal surgery. First, their common bile duct (CBD) was ligated to cause cholestasis by total biliary obstruction (TBO). On day 0, 1, 3, 7, 14, 21 and 28 after TBO, magnetic resonance imaging (MRI) was conducted to monitor the dilatation of the CBD, and blood was sampled to analyze total serum bilirubin (TSB). Secondly, on day 30, the abdomen was re-opened and gallstone(s) collected from human patients were implanted in the dilated CBD as



a virtual gallbladder (VGB), which was closed by suture ligation. This rat cholelithiasis model was examined by MRI, clinical observation, microcholangiography and histology.

**RESULTS:** All rats survived two laparotomies. After ligation, the CBD was dilated to a stable size of 4 to 30 mm in diameter on day 21-28, which became a VGB. The rats initially showed signs of jaundice that diminished over time, which paralleled with the evolving TSB levels from  $0.6 \pm 0.3$  mg/dL before ligation, through a peak of  $10.9 \pm 1.9$  mg/dL on day 14, until a nearly normalized value after day 28. The dilated CBD with thickened wall allowed an incision for implantation of human gallstones of 1-10 mm in diameter. The rat cholelithiasis was proven by *in vivo* MRI and postmortem microcholangiography and histomorphology.

**CONCLUSION:** A rat model cholelithiasis with human gallstones has been established, which proves feasible, safe, reliable, nontoxic and cost-effective. Given the gallstones of human origin, applications of this model may be of help in translational research such as optical detection and lysis of gallstones by systemic drug administration.

**Key words:** Cholelithiasis; Rat; Gallbladder; Common bile duct; Cholestasis; Bilirubin; Gallstones

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**Core tip:** The mouse and rat are common experimental animals. Unlike the mouse that has a gallbladder but difficulty in imaging studies, the rat does not have a gallbladder, which has hampered studies on imaging gallstones. To tackle this problem, we first induced a virtual gallbladder (VGB) in rats by ligation of the common bile duct (CBD) accompanying with gradual increase and normalization of serum bilirubin. Then we implanted gallstone(s) collected from human patients into the dilated CBD or VGB to create a cholelithiatic model in rats, which has been validated by *in vivo* magnetic resonance imaging, microcholangiography and histology. This rat model is deemed useful for translational research such as fluorescent visualization of gallstones for laparoscopic detection and differential diagnosis of cholelithiasis.

Miranda Cona M, Liu Y, Yin T, Feng Y, Chen F, Mulier S, Li Y, Zhang J, Oyen R, Ni Y. Rat model of cholelithiasis with human gallstones implanted in cholestasis-induced virtual gallbladder. *World J Methodol* 2016; 6(2): 154-162 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i2/154.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i2.154>

## INTRODUCTION

Cholelithiasis refers to the presence of gallstones that

are concreted from bile components in the biliary tract, usually in the gallbladder. Cholelithiasis is a clinical syndrome<sup>[1-3]</sup> with a global incidence in up to 20% of the adult population<sup>[2,4,5]</sup>. The risk factors for developing gallstones include gender, age, geographic location, genes, ethnicity, metabolic conditions, obesity, pregnancy, diet and alcohol or drug consumption<sup>[6,7]</sup>. Cholelithiasis can trigger acute cholecystitis or gallbladder inflammation leading to severe abdominal pain, jaundice and secondary infections by intestinal microorganisms<sup>[8,9]</sup>. More life-threatening complications include acute cholangitis or pancreatitis<sup>[10,11]</sup>. Stones larger than 3 cm or gallbladders packed with stones might also increase the risk of gallbladder cancer<sup>[6]</sup>.

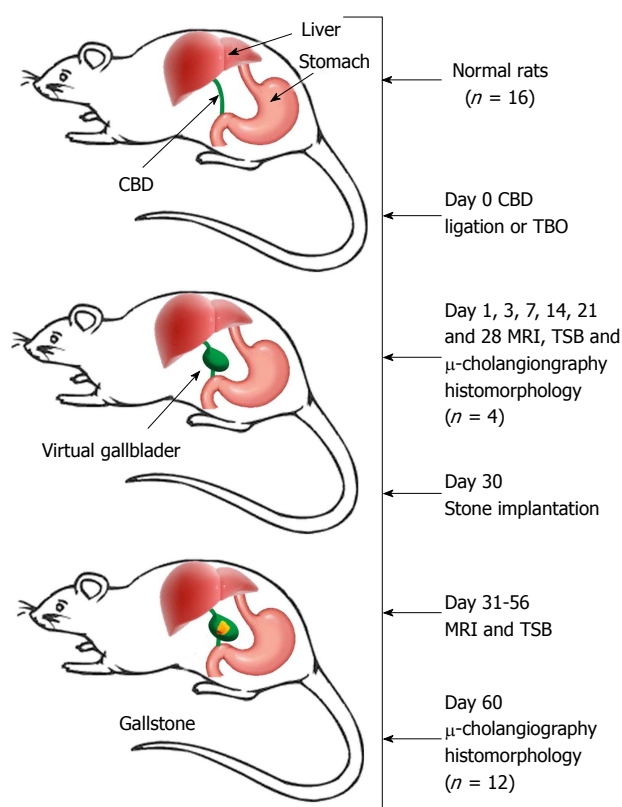
Over the past decades, research on diagnosis, prevention and treatment of cholelithiasis has been a popular focus of medical community. By using different methods including gallbladder infection, induced biliary stasis, and modified diet<sup>[12]</sup>, animal models of cholelithiasis have been created to resemble human pathophysiology. However, these models are often cost-ineffective and unreliable with a low rate of gallstone formation or marked hepatotoxicity<sup>[12,13]</sup>. In addition, their gallstone compositions may differ from that in humans, making it difficult to extrapolate experimental findings to clinic applications.

Rodents including mice and rats are inexpensive and widely applied experimental animals. Rats are 10-fold bigger in dimension than mice, which eases many of the research procedures including noninvasive imaging diagnosis using clinical scanners<sup>[14]</sup>. But, comparing to mice, rats lack a gallbladder by nature<sup>[12]</sup>, which otherwise would make it impossible to research cholelithiasis in this species. However, based on its unique biliopancreatic features<sup>[15]</sup>, we hypothesized that it could be feasible in the rat first to create a hollow biliary organ as a virtual gallbladder (VGB), and then to implant human gallstones in it without compromising normal bile flow at 22.5 mL/d<sup>[16]</sup>. To test this hypothesis, we conducted two phases of abdominal surgery to ligate and dilate the common bile duct (CBD) first and, one month later, to implant gallstones in order to create a rat model of cholelithiasis. We further validated this model by *in vivo* magnetic resonance imaging (MRI), serial blood bilirubin tests, postmortem microcholangiography, and histomorphological examinations. Potential implications of this rat model of human cholelithiasis for translational research include fluorescent visualization of gallstones for laparoscopic detection and differential diagnosis of gallstones after systemic administration of small molecular drugs that undergo hepatobiliary excretion as being proven preliminarily<sup>[17]</sup>. Similarly, new litholytic chemicals can be tested this way for therapy.

## MATERIALS AND METHODS

### Animals

Twenty adult Wistar rats of equal genders weighing 300-350 g were purchased from Charles River La-



**Figure 1** Flow chart of the experimental procedures. CBD: Common bile duct; MRI: Magnetic resonance imaging; TBO: Total biliary obstruction; TSB: Total serum bilirubin;  $\mu$ -cholangiography: Microcholangiography.

boratories, Inc. (St. Aubain les Elbeuf, France) and housed in environmentally controlled conditions including temperature at  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and relative humidity at  $60\% \pm 10\%$  on a 12:12 h light-dark cycle. Standard rat chow (Ssniff Spezialdiäten GmbH, Soest, Germany) and water were available ad libitum. All experimental procedures were approved by the institutional Animal Ethics Committee and complied with the European Ethics Committee guidelines (decree 86/609/EEC). As illustrated in Figure 1, eventually 16 rats were included after pilot tests in the first 4 rats.

### Creation of a rat model of cholelithiasis

**Ligation of CBD to create a VGB:** Under anesthesia with intraperitoneal injection of sodium pentobarbital (Nembutal; Sanofi Sante Animal, Brussels, Belgium) at 40 mg per kilogram body weight and after shaving and sterilizing the skin, a midline abdominal incision of 4-5 cm was made through the skin, muscle and peritoneum (Figure 2A). The stomach and duodenal loop were exposed, and the CBD was identified underneath the hepatic hilum. The CBD was ligated adjacent to the duodenum<sup>[15,18]</sup> using a 5-0 silk suture (Ethicon, New Jersey, United States). The abdominal wall was closed with two layers of consecutive sutures and animals were allowed for recovery from anesthesia and surgery. The rat model of VGB was thus initiated (Figure 2A-2D).

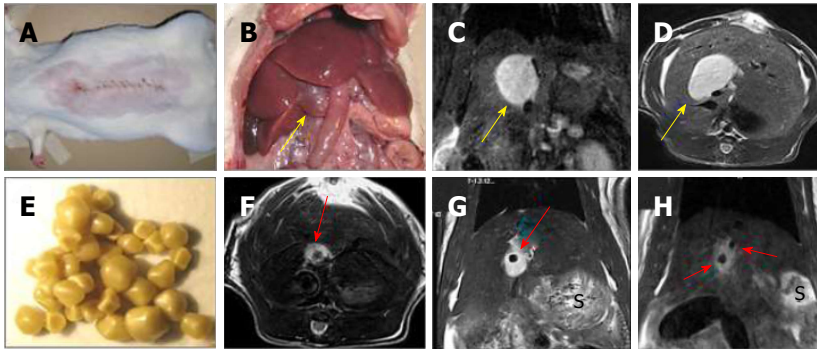
**Implantation of human gallstones in the VGB to create a cholelithiasis model:** Gallstones of cholesterol, pigment and mixed contents of 1-10 mm in diameter were collected from patients after cholecystectomy (Figure 2E). Stones were disinfected by immersion in 70% ethanol for 5 min and rinsed with sterile physiological saline. Thirty days following CBD ligation, under the same anesthetic and laparotomic procedures as in the first surgery, the abdomen of CBD-ligated rats was reopened. The VGB was identified and the yellowish bile was aspirated using a syringe with a 27 gauge needle. The needle hole was extended to a length 5 mm by using a pair of microsurgical scissors. Depending on the actual size of the VGB and the purpose of the study, 1-3 gallstones could be inserted into the cavity (Figure 2F-2H). The opening was then closed with a suture ligation and the animal was left to recover from the surgery.

### Assay of total serum bilirubin

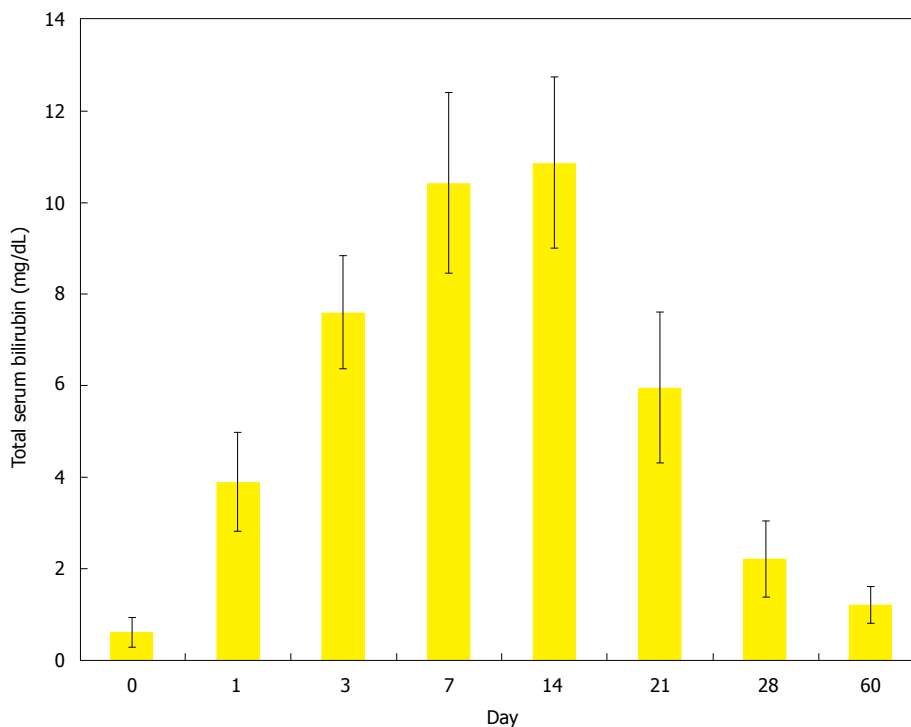
For all animals, serum bilirubin was determined on the day before of the CBD ligation and on day 1, 3, 7, 14, 21, 28 and 60 after operation (Figures 1 and 3). Fresh blood samples were collected in VACUETTE® heparin tubes by cutting a small section of the distal tail with a fresh scalpel blade. A droplet of Histoacryl® topical adhesive (Braun, Tuttlingen, Germany) was applied on the skin incision to stop bleeding. For serum separation, the blood samples were allowed to coagulate in the dark to prevent enzyme degradation. The samples were then centrifuged at 4000 rpm for 10 min at  $4^{\circ}\text{C}$  using a 4226 ALC Centrifuge (Milan, Italy). The supernatant containing the serum was transferred to 1.5 mL micro-centrifuge Eppendorf® Safe-Lock® vials. With a Hitachi automatic analyzer (Tokyo, Japan), analysis of total serum bilirubin (TSB) was carried out.

### MRI of the VGB and cholelithiasis

Before and after each surgical procedure, all rats underwent serial MRI scans to monitor the progressive expansion of the CBD (Figure 2C and 2D) and to detect the presence of gallstone(s) in the VGB (Figure 2F-2H, Figure 4A and Figure 5A). Briefly, under gas anesthesia using 2% isoflurane (Halocarbon, River Edge, NJ, United States) mixed with 20% of  $\text{O}_2$  and 80% of air, MRI was conducted at a clinical 3.0T whole body MR magnet (Trio; Siemens, Erlangen, Germany) with a maximum gradient capability of 45 mT/m with an 8-channel phased array wrist coil (*in vivo*, Latham, NY, United States). T2 weighted turbo spin echo images were acquired at both transverse and coronal planes of 1 mm thickness with the following parameters as TR/TE = 4300/69 ms, flip angle =  $140^{\circ}$ , turbo factor = 13, field of view = 77 mm  $\times$  85 mm, matrix = 414  $\times$  512, number of averages = 6 and total imaging time of 3 min 9 s.



**Figure 2** Creation of a rat model of cholelithiasis. A: Midline abdominal incision for CBD ligation; B: Laparotomy to expose the cholestasis-induced VGB (arrow); C: Coronal plane of T2-w MRI displaying an oval-shaped hyperintense dilated CBD or a VGB (arrow); D: Axial plane of T2-w MRI displaying the same oval-shaped hyperintense VGB (arrow); E: Gallstones from patients of cholelithiasis derived by cholecystectomy; F: Axial plane of T2-w MRI showing the same case in D but now implanted with a human gallstone (arrow); G: Coronal plane of T2-w MRI displaying the same case in F with a gallstone (arrow); H: Coronal plane of T2-w MRI two hypointense gallstones (arrows) located in a hyperintense VGB in another case. S denotes stomach on MR images. CBD: Common bile duct; VGB: Virtual gallbladder; MRI: Magnetic resonance imaging.



**Figure 3** Evolving changes of total serum bilirubin levels in rats after ligation of common bile duct to induce a rat model of virtual gallbladder and cholelithiasis. Total bilirubin levels reached the highest values between the days 7 and 14 that and then rapidly dropped to the values close to the normal range after day 28 till day 60.

### Postmortem microcholangiography and histomorphology

Rats were euthanized by intravenous injection of an overdosed Nembutal. The abdomen was opened with a skirt incision to expose all abdominal visceral organs (Figure 2B). Using a method similar to that reported previously<sup>[18]</sup>, the VGB without or with implanted gallstone(s) was punctured with a needle-syringe to aspirate the bile. Barium sulphate suspension (Micropaque; Guerbet, France) was injected to fill in the cavity, which was closed by suture ligation. The hepatic, biliopancreatic and duodenum were entirely excised and digitally radiographed (Mammomat Inspiration;

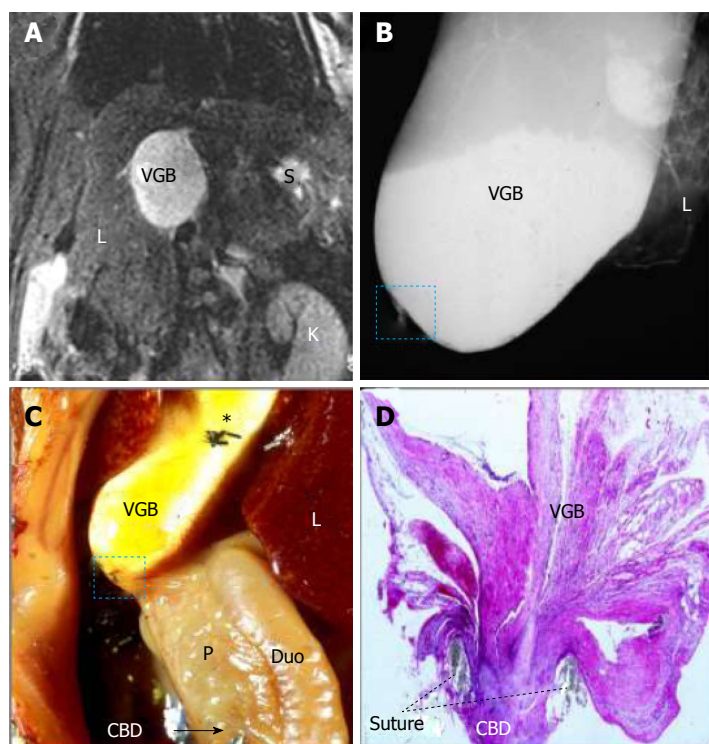
Siemens, Germany) at 26 kV and 15 mA (Figure 4B and Figure 5B), followed by fixation in 10% formaldehyde for histomorphology (Figure 4C, 4D and Figure 5C, 5D).

## RESULTS

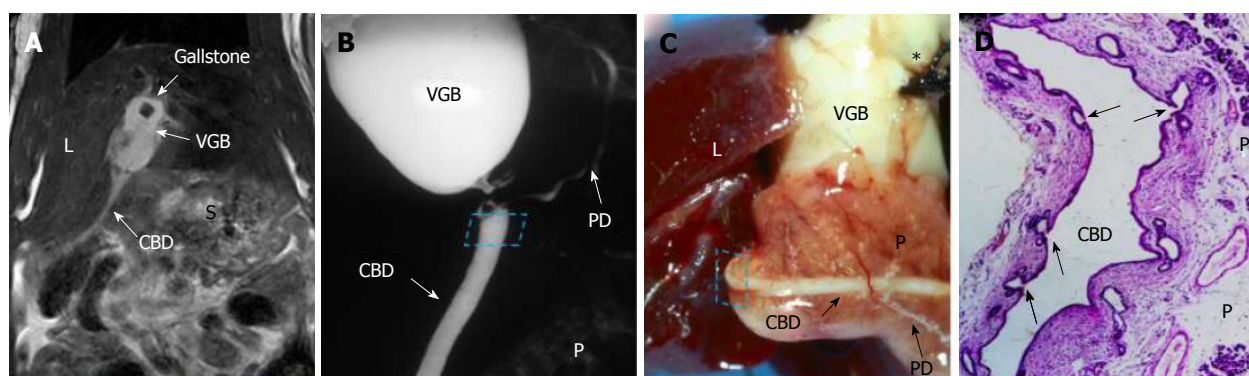
### General conditions

All 20 rats tolerated well the anesthesia and surgery. However, during early pilot stage, 4 rats failed to form the final cholelithiasis model, one rat died of cholestasis before bile flow recovery, one rat did not show CBD dilatation probably due to invalid obstruction because of a too distal ligation site, and other two died of





**Figure 4** Magnetic resonance imaging, microcholangiography and histomorphology of a rat on day 14 after surgically induced cholestasis towards creating a model of virtual gallbladder. A: Coronal T2-w MRI shows an oval-shaped hyperintense dilated CBD or VGB with L, K and S denoting the liver, kidney and stomach respectively; B: Digital microcholangiography displays a hyperdense dilated CBD or VGB with L denoting the liver; C: Laparoscopic view shows the dilated CBD or VGB with L, P and Duo denoting the liver, pancreas and duodenum respectively; note the transparent distal CBD as indicated by a needle tip and an arrow, suggesting absent bile flow, and asterisk indicates where the needle hole was closed by suture ligation; D: Photomicrograph of hematoxylin and eosin stained slide of the ligature (dashed square on B and C) shows a complete CBD obstruction separating the VGB and distal CBD (original magnification  $\times 100$ ). MRI: Magnetic resonance imaging; CBD: Common bile duct; VGB: Virtual gallbladder.



**Figure 5** Magnetic resonance imaging, microcholangiography and histomorphology of a rat on day 60 after surgically induced cholelithiasis. A: Coronal T2-w MRI shows a hypointense gallstone located in a hyperintense VGB and distal nondilated CBD as a hyperintense line with L and S denoting the liver and stomach; B: Digital microcholangiography displays both the hyperdense VGB and distal nondilated CBD bridged in-between by a pancreatic ductule with P denoting the pancreas; C: Macroscopic view shows the VGB with L denoting the liver, note the distal nondilated and barium-filled CBD imbedded in the pancreas (P) and a branching ductule bridging the ligation between the proximal and distal CBD, suggesting resumed bile flow, asterisk indicates where the needle hole was closed by suture ligation; D: Photomicrograph of hematoxylin and eosin stained slide of the distal CBD near the ligature (dashed square on B and C) demonstrates multiple proliferated glandular ductules (arrows) that contribute to the eventual bypass of the biliary obstruction between the VGB and distal CBD (original magnification  $\times 100$ ). MRI: Magnetic resonance imaging; CBD: Common bile duct; VGB: Virtual gallbladder; PD: Pancreatic duct.

biliary peritonitis due to the bile leakage after needle suture, instead of suture ligation, to close the incision after gallstone implantation. Thus, the remaining 16 rats were included in this study (4 for cholestasis only and 12 for cholelithiasis) and sacrificed at designated time points for postmortem examinations. No gender

difference was noticed regarding experimental procedures and outcomes.

#### Rat model of VGB

A VGB was successfully established by ligation of CBD, which proved to be safe and was confirmed by *in vivo*



**Table 1** Magnetic resonance imaging measurements and drained bile volumes from rats with cholestasis ( $n = 4$ ) and cholelithiasis ( $n = 12$ )

No. of rats	VGB diameter (cm)	Bile volume (mL)	Incidence (%)
3	3.1-4.0	$7.2 \pm 1.9$	18.8
6	2.1-3.0	$3.8 \pm 1.4$	37.5
5	1.1-2.0	$2.6 \pm 1.2$	31.3
2	0.5-1.0	$0.8 \pm 0.3$	12.5

VGB: Virtual gallbladder.

MRI, normalized total bilirubin, microcholangiography and histology (Figures 2-5). The entire procedure from induction of anesthesia to closure of skin incision took approximately 15 min. During the first 3 wk after surgery, the animals showed signs of jaundice including yellowish skin and sclera as well as whitish feces, which worsened on day 7 to 14 but gradually diminished afterwards. The CBD-ligated rats maintained fair life quality with normal physical/behavioral manifestations without apparent loss of body weight. As shown in Table 1, a wide diversity in the dimension of VGB was found, ranging from 0.5 to 4.0 cm in diameter and the collected bile juice varied from 0.8 to 7.2 mL.

#### Rat model of cholelithiasis

All the 12 rats survived the anesthesia and surgical procedures for gallstone implantation in the VGBs. Each surgery took about 30 min, somewhat longer than the first operation due to intraperitoneal adhesion. After VGB isolation and bile aspiration, the gallstone(s) were successfully implanted, as seen on MRI (Figure 2F-2H and Figure 5A). No complications such as bleeding, enterocutaneous fistula, infection or sepsis, tissue damage or organ injury were observed. Minimum intra-abdominal adhesions between the liver and intestinal loops near the VGB were encountered, which were however manageable by blunt separation maneuver. Altogether 20 gallstones varying 1-10 mm in size were implanted, *i.e.*, 3, 2 and 1 stone(s) in 2, 4 and 6 rats respectively.

#### MRI findings

On coronal and transverse T2-w MRI, 2-3 wk after ligation, a dilated CBD or VGB formed a hyperintense hollow organ of pear, circular or cylindrical shapes located under the liver at the right upper quadrant region of the abdomen, simulating the organ of gallbladder (Figures 2C, 2D, 4A and 5A). The patent distal CBD was also occasionally visible (Figure 5A). T2-w MRI of this rat model of cholelithiasis typically displayed hypointense gallstone(s) located in a hyperintense dilated CBD or VGB (Figure 2F-2H and Figure 5A).

#### Serum biochemical parameters

Figure 3 depicts the changes of the serum total bilirubin concentrations in function of time from CBD-ligated rats on the day before the surgery for CBD ligation and on

day 1, 3, 7, 14, 21, 28 and 60 after operation. Before CBD ligation, the total bilirubin values were averaged at a normal level of  $0.6 \pm 0.3$  mg/dL. On the successive days after surgery, these values increased up to  $10.9 \pm 1.9$  mg/dL on day 14, but rapidly declined afterwards to the level close to the normal range, *i.e.*,  $2.2 \pm 0.8$  mg/dL and  $1.2 \pm 0.4$  mg/dL on day 28 and 60, respectively.

#### Microcholangiographic findings

On digital microcholangiography, the dilated CBD or VGB appeared hyperdense due to the instilled barium suspension (Figures 4B and 5B). The 4 rats with hyperbilirubinemia or cholestasis sacrificed before day 21 failed to display the opacified distal CBD (Figure 4B), whereas all 12 rats of cholelithiasis displayed both the VGB and patent distal CBD due to anastomosis formation between biliopancreatic ducts (Figure 5B).

#### Histomorphological findings

At autopsy, the dilated CBD appeared as a hollow organ filled with the bile that was substituted by barium sulphate suspension for microcholangiography, suggestive of a VGB (Figures 4C and 5C). Initially there was no bile flow in the distal CBD due to ligation induced cholestasis (Figure 4C), but with time the biliary obstruction was resolved by collateral formation (Figure 5C). Microscopically, while the CBD was completely obstructed in certain cases (Figure 4D), in other cases multiple proliferated biliary ductules could be found around the CBD and pancreatic tissue, which bypassed the ligation (Figure 5D). These findings were consistent with those from MRI (Figure 5A) and microcholangiography (Figure 5B).

## DISCUSSION

Cholelithiasis refers to the gallstone formation in the biliary tract, usually in the gallbladder. Animal models of biliary infection and cholestasis have been applied in research for etiology and diagnosis of cholelithiasis as well as drug intervention and diet modification for the treatment of the disease. These models are often poorly manageable, inefficient, expensive, unreliable, and hepatotoxic or their applications are hardly translational to human scenarios. In the present study, we have strived to establish and validate a rat model of human gallstones potentially useful in research on cholelithiasis or choledocholithiasis especially for developing effective diagnostic and therapeutic techniques. This rat model of cholelithiasis can be realized by two sequential steps: (1) creating a VGB in rats by chronic expansion of the CBD into a cystic cavity after CBD ligation; and (2) implanting human gallstone(s) in the VGB with resumed biliary flow and efficient intestinal discharge, as proven by the *in vivo* MRI findings (Figure 5A), serum bilirubin tests (Figure 3), microcholangiography (Figure 5B) and histomorphology (Figure 5C and D). Overall, this rat model of cholelithiasis turned out to be feasible for

preparations and reliable for validation. Recently a novel optical imaging agent for diagnosis and differential diagnosis of human gallstones has been identified by using this animal model (paper submitted).

Unlike experimental gallstone models in the previous studies conducted on gallbladder-bearing species such as ground squirrels<sup>[19]</sup>, mice<sup>[20]</sup>, dogs, cats, guinea pigs, rabbits, sheep, pigs and cattle<sup>[12]</sup>, our experiments were conducted on rats that lack a gallbladder. The rat specie was chosen due to its amplest availability, lowest cost, easiest housing and handling, in addition to its feasibility of being imaged by using clinical scanners<sup>[14]</sup>. However, because of its natural deficiency<sup>[12]</sup>, a structure resembling a gallbladder in both morphology and functionality with rats deems mandatory to further study cholelithiasis. In concordance with previous studies<sup>[17,21]</sup>, after ligation the proximal CBD increased its diameter with a noticeably thickened wall due to the evolving bile stasis. Studies showed that the diameters of the CBD could enlarge from 0.3 to 8 mm in only 5 d owing to bile accumulation<sup>[21]</sup>, and further dilate to a few centimeters in 2-3 wk<sup>[18]</sup>. This can explain the dimensions up to 40 mm of the VGB with a large volume of bile collected in our model rats 4 wk after CBD ligation.

A recovery period of four weeks was chosen, because shortly after surgery the rats showed limited activity with weak mechanical strength of the CBD. In animals, persistent obstruction of CBD may cause cholestatic liver injury, hyperbilirubinemia, portal hypertension, ascites, endocrine disorders, hepatic encephalopathy and skeletal fragility (osteopenia)<sup>[22-24]</sup>. Interestingly, especially in rats, after a course of about 4 wk of CBD obstruction, they progressively recovered from cholestasis with a diminishing serum bilirubin level, improved jaundice symptoms, and gain in body weight and strength. This recovery response could be explained by the unique proliferative changes of the biliopancreatic duct tree after a complete CBD-ligation in rats<sup>[18,25]</sup>. Contrary to humans, in which complete biliary obstruction can be life threatening<sup>[26]</sup>, most of such cholestatic rats eventually survived due to formation of sufficient biliary collaterals, which has been widely referred in the literature<sup>[18,25,27]</sup>. Indeed, the CBD-ligated rats have been used as a model of typical and selective cholangiocytic proliferation, which is characterized by an increased biliary pressure that triggers newly formed capillary networks or peribiliary capillary plexuses surrounding the intrahepatic bile duct branches over time<sup>[27,28]</sup>. It seems that native bile ducts, the preexisting bile capillary and proliferated ductules jointly play a significant role in the transport mechanism of biliary substances, secretion and reabsorption of water and electrolytes<sup>[29]</sup>. Therefore, this phenomenon might explain the recovery of the CBD-ligated rats during thirty days after a complete CBD obstruction in rats. Moreover, a period of thirty days also allowed the natural absorption of the intra-abdominal fibrous adhesion caused by the first CBD-ligation surgery. As a consequence, during the second surgery for stone

implantation, the exposure of and access to the newly formed VGB became easier with less complications. Furthermore, during this elongated period, the thickened "gallbladder" wall due to tissue proliferation eased incision and closure for stone implantation and prevention of bile leakage.

The second step for preparing the rat model of cholelithiasis is to embed human gallstone(s) into the VGB of rats 4 wk after CBD-ligation. Human gallstones were categorized into 3 common types as cholesterol, pigment and mixed stones<sup>[30]</sup>. Therefore, the rat cholelithiasis model introduced here would be suitable in studies on diagnostic or therapeutic interventions with chemical drugs that could undergo the bile excretion and further interact with a particular component of the stone from human patients as exemplified in our recent study<sup>[17]</sup>.

By direct implantation of human gallstones, we intended to overcome the limitations with the previous models of gallstone disease such as gallbladder infection, production of biliary stasis, and diet composition modifications<sup>[12]</sup>. Gallbladder concretions were generated in rabbits subjected to infection with different bacteria such as coliform, vibrio cholera, typhoid bacilli, and streptococci isolated from tonsils and human gallbladders<sup>[12,31]</sup>. Biliary stasis based on CBD ligation in combination with bacteria inoculation resulted in more reliable formation of gallstone than bacterial injection alone<sup>[32]</sup>. Gallbladder trauma along with bacterial infection has been also proved to be effective to cause gallstones<sup>[33]</sup>. However, these bacterial methods generally require serial inoculations and longer time. In the case of the bile stasis methods, they could be unreliable and inconsistent. The gallstone production by dietary modifications appeared successful with numerous dietary schemes including cholesterol and sunflower seed oil in rabbits<sup>[12]</sup>, cholesterol rich foods<sup>[34]</sup>, diet rich in sucrose (74.3%) with low lipid content in hamsters<sup>[35]</sup>, and ground chow either with 1% cholesterol or 0.5% cholic acid in mice<sup>[36]</sup>. However, the dietary methods were frequently hepatotoxic and some animals poorly tolerated the diet, and the models were time consuming and inconsistent<sup>[12]</sup>. The most rapid and reliable system seemed to be the dihydrocholesterol-rabbit model leading to cholelithiasis within fifteen days without apparent toxicity in the liver. However, the gallstone composition was dissimilar to that in humans, thus not translational for many applications. The formation of human-type gallstones by feeding guinea pigs with modocoll and cholestyramine was also tried with satisfactory frequency and quickness without hepatotoxicity<sup>[12]</sup>, which however was more expensive than other small rodent models<sup>[13]</sup>.

There exist advantages and disadvantages or limitations with this cholelithiasis model in rats. First, the model was not intended for studying the formation or etiology of gallstones, instead it is for validating diagnostic or therapeutic agents potentially interactive with human gallstones after systemically administration,

hepatic uptake or metabolism and biliary excretion. Second, the model appears complex to make, but all rats tolerated the twice surgical procedures with a high success rate. Third, indeed the ligation of the CBD induced cholestatic liver injury, cholangiocellular proliferation, ductular reaction and fibrosis up to the development of biliary cirrhosis from seven days to weeks after surgery. But, in rodents bile flow does resume with time as proven by gradually normalized serum hepatic enzymatic parameters<sup>[18]</sup>, which closely simulates cholelithiatic patients with chronic and repeated onset of cholestasis. Thus, this rat model of human gallstones with background histological alterations could be suitable for certain clinically relevant translational research<sup>[17]</sup>.

Technically, the high success rate of this model was assured by two major factors. First, the cholestasis induced by CBD ligation in rats is only transient as evidenced by normalization of serum bilirubin level and resumed bile flow seen on micro-cholangiography<sup>[18]</sup>. Secondly, after implantation of human gallstones, careful closure of VGB wall proved crucial to avoid any bile leakage that is a lethal complication.

In conclusion, we have introduced a rat model of cholelithiasis with human gallstones, which appears to be an experimental model with certain desirable characteristics<sup>[12]</sup>. It is a relatively simple and cost-effective method that offers the possibility of studying already formed human gallstones in rats. It was not toxic since no special diet different to the standard rodent chow is required. Another practical aspect is the time consumed for the model of gallstones. In contrast to previous reports in which the formation can occur in up to a year<sup>[12]</sup>, the proposed animal model can be invariably ready by around sixty days. Most importantly it may offer translational results for clinically relevant applications since it contains human gallstones.

## COMMENTS

### Background

The clinical syndrome cholelithiasis refers to the presence of gallstones in the biliary tract, usually in the gallbladder, with a global incidence in up to 20% of the adult population. The risk factors for developing gallstones include gender, age, geographic location, genes, ethnicity, metabolic conditions, obesity, diet and alcohol or drug consumption. Cholelithiasis can trigger acute cholecystitis leading to severe abdominal pain, jaundice and secondary infections as well as more life-threatening complications include acute cholangitis and pancreatitis with increased risk of gallbladder cancer.

### Research frontiers

Over the past decades, research on diagnosis, prevention and treatment of cholelithiasis has been a popular focus of medical community. By using different methods including gallbladder infection, induced biliary stasis, and modified diet, animal models of cholelithiasis have been created to resemble human pathophysiology. However, these models are often cost-ineffective and unreliable with a low rate of gallstone formation or marked hepatotoxicity. In addition, their gallstone compositions may differ from that in humans, making it difficult to extrapolate experimental findings to clinic applications.

### Innovations and breakthroughs

Both the mouse and the rat are the most common experimental animals.

However, unlike the mouse that has a gallbladder but has more difficulties in imaging studies, the rat by nature does not have a gallbladder, which has hampered research on imaging gallstones. To tackle this problem, the authors first induced a virtual gallbladder in rats by ligation of the common bile duct. Then they implanted gallstone(s) collected from human patients into the virtual gallbladder to create a cholelithiatic model in rats, which was validated by *in vivo* magnetic resonance imaging and evolving serum bilirubin levels, and *ex vivo* microcholangiography and histology.

### Applications

This rat cholelithiasis model is deemed useful in certain translational research such as fluorescent visualization of gallstones for laparoscopic detection and differential diagnosis, as well as in studying cholelithiolysis by chemicals that are systemically administered, taken up by hepatocytes and excreted via the bile.

### Peer-review

This is an original research paper describing the methodology for establishing and validating a rat model of cholelithiasis, which therefore falls well into the scope of this journal.

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## Case Control Study

# Mild oxidative stress is beneficial for sperm telomere length maintenance

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## Abstract

**AIM:** To evaluate telomere length in sperm DNA and its correlation with oxidative stress (normal, mild, severe).

**METHODS:** The study included infertile men ( $n = 112$ ) and age matched fertile controls ( $n = 102$ ). The average telomere length from the sperm DNA was measured using a quantitative real time PCR based assay. Seminal reactive oxygen species (ROS) and 8-Isoprostane (8-IP) levels were measured by chemiluminescence assay and ELISA respectively.

**RESULTS:** Average sperm telomere length in infertile men and controls was  $0.609 \pm 0.15$  and  $0.789 \pm 0.060$ , respectively ( $P < 0.0001$ ). Seminal ROS levels in infertile was higher [ $66.61 \pm 28.32$  relative light units (RLU)/s/million sperm] than in controls ( $14.04 \pm 10.67$  RLU/s/million sperm) ( $P < 0.0001$ ). The 8-IP level in infertile men was significantly higher ( $421.55 \pm 131.29$  pg/mL) than in controls ( $275.94 \pm 48.13$  pg/mL) ( $P <$

0.001). When correlated to oxidative stress, in normal range of oxidative stress (ROS, 0-21.3 RLU/s/million sperm) the average telomere length in cases was  $0.663 \pm 0.14$ , in mild oxidative stress (ROS, 21.3-35 RLU/s/million sperm) it was elevated ( $0.684 \pm 0.12$ ) and in severe oxidative stress (ROS > 35 RLU/s/million sperm) average telomere length was decreased to  $0.595 \pm 0.15$ .

**CONCLUSION:** Mild oxidative stress results in lengthening of telomere length, but severe oxidative stress results in shorter telomeres. Although telomere maintenance is a complex trait, the study shows that mild oxidative stress is beneficial in telomere length maintenance and thus a delicate balance needs to be established to maximize the beneficial effects of free radicals and prevent harmful effects of supra physiological levels. Detailed molecular evaluation of telomere structure, its correlation with oxidative stress would aid in elucidating the cause of accelerated telomere length attrition.

**Key words:** Telomere; Oxidative stress; Reactive oxygen species; Infertility; 8-Isoprostane

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**Core tip:** In the present study we found that infertile men experienced oxidative stress evident from increased seminal reactive oxygen species and 8-Isoprostane levels. Infertile men also had shorter telomeres as compared to the controls. Severe oxidative stress negatively affected sperm telomere length but surprisingly mild oxidative stress resulted in lengthening of telomere and thus may aid in maintaining genomic integrity. To the best of our knowledge we are the first to report positive effect of mild oxidative stress on sperm telomere length.

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## INTRODUCTION

Infertility is a complex life style disorder and affects about 1 in 5 couples. Oxidative damage to sperm DNA is one of the major causes of infertility especially in men with normal sperm parameters. Recent studies have shown that sperm DNA integrity, mitochondrial genome, gene expression, and sperm telomere length along with the interplay of more than 2000 genes and gene environment interaction are the key players in male infertility. Studies have shown that oxidative stress is a major player in male infertility. Oxidative stress affects all the bio molecules, membrane lipids, nuclear and mitochondrial genome. Various studies have explored

the role of oxidative stress and DNA damage in male infertility but there are few studies to understand the role of sperm telomeres and its length in the aetiology of male infertility. Shorter telomeres may result in impaired spermatogenesis and fewer cell divisions which may ultimately manifest as oligozoospermia or azoospermia.

Telomeres are hexameric guanine rich repeats present at the ends of chromosomes in all eukaryotic cells<sup>[1]</sup>. Human telomeres are composed of long stretches of the repetitive sequence TTAGGG and a telomere-specific protein complex, Shelterin. TRF1, TRF2 and POT1 are the three proteins that impart Shelterin the specificity for telomeres<sup>[2]</sup>. TIN2 and TPP1 link POT1 to TRF1 and TRF2. The t-loop of the telomeric lariats are formed due to the invasion of the 3' single-stranded overhang into the double-stranded telomeric DNA. Shelterin caps most of the double stranded telomeric region and POT1 covers the single-stranded telomeric DNA in the 3' overhang or in the D loop. Various shelterin associated proteins and nucleosomes are also found associated to telomeric DNA<sup>[3]</sup>.

In mammalian sperm nuclei, all telomeres exist in the form of dimers. In humans, these dimers are localized at the nuclear periphery and, most probably, interact with the nuclear membrane. The human telomere sequence varies in length from 5 to 10 kb in somatic cells and 10 to 20 kb in germ cells. Telomere length is critical for chromosome stability, cell proliferation and survival. Telomere length in spermatozoa is substantially longer compared to normal somatic cells<sup>[4]</sup>. Initial telomere length setting happens in the sperm cells before fertilization. Sperm cells are the carriers of intact chromosomes to the progeny. Yet only limited reports are available regarding the role of telomere dynamics in germ cells.

During cell division telomeres are not fully replicated because of end replication problem, an inability of the DNA polymerase to completely replicate the DNA ends resulting in telomere shortening with every cell division. Telomerase, an enzyme consisting of the telomerase RNA template and telomerase reverse transcriptase maintains telomere length by adding nucleotide repeats to the chromosome ends.

Other causes of telomere shortening apart from normal cell division include oxidative stress, genotoxic insults, and genetic predisposition. Telomere shortening has been associated with oxidative stress<sup>[5]</sup>. Reactive oxygen species (ROS) resulting endogenously from normal cellular metabolism and due to exogenous genotoxic insults like environmental exposure, exposure to electromagnetic radiations, exhaustive exercise, poor life style habits also shorten telomeres by oxidizing guanine residues in the telomeric DNA and thereby initiating a DNA damage response. DNA damage response results in excision of telomere repeats<sup>[6]</sup>. The high content of guanine nucleotides - in telomeric DNA makes telomere a preferred target for oxidative damage<sup>[7]</sup>. Thus this study was planned with an aim

to study impact of oxidative stress on telomere length. Though sperm maintain longer average telomere length, sperm telomere length does vary among individual men and individual spermatozoa. Variability in sperm telomere length is due to telomere shortening as a result of differential telomerase activity and oxidative stress<sup>[8]</sup>. Cellular environment also plays an important role in regulating telomere length and telomerase activity. Most notably, oxidative stress can shorten telomeres and antioxidants can decelerate shortening<sup>[9]</sup>. Studies by Kumar *et al.*<sup>[10,11]</sup> have shown upregulation in telomerase activity and decline in free radical levels, oxidized mutagenic bases following practice of yoga and meditation. Telomere shortening in sperm cells results in segregation errors, generation of unbalanced gametes, reduced sperm count due to apoptosis which ultimately leads to loss of fertility potential and abortions. There are few studies on the role of telomere in reproduction and since oxidative stress has such a marked effect on telomere phenotype, it is important to evaluate the effect of oxidative stress (normal, mild, severe) on telomere length. It is important to establish cut off levels of free radicals which are beneficial to telomeres and cause telomere lengthening as compared to very high ROS levels which induce DNA damage response in telomeres and cause telomere shortening. In this study we found longer telomere length in cases with mild oxidative stress levels [21.3-35 relative light units (RLU)/s/million sperm] as compared to cases with normal ROS levels (0-21.3 RLU/s/million sperm). Thus it would be more valuable to establish cut off free radical levels which are beneficial/detrimental to telomere length maintenance. In this study we have investigated the role of oxidative stress in sperm telomere length maintenance in idiopathic male infertility.

## MATERIALS AND METHODS

The study was initiated after institutional ethical clearance and written informed consent from patients and controls. The female partners of all the cases were normal after complete clinical, gynaecological, hormonal and radiological examination. Human ejaculates were obtained from 102 healthy volunteers of proven fertility from family planning OPD of Obstetrics and Gynaecology Department, and 112 male partners of couples experiencing primary infertility within age group of 18-45 years. A detailed family history was recorded in a pre-designed proforma. Cytogenetic analysis was done for all cases to exclude cases with abnormal chromosome complement. All cases with recent history of fever, drug intake, any inflammatory disorders, or infections were excluded. Semen analysis was assessed by World Health Organization (WHO) (1999) criteria. These patients after thorough clinical examination were referred from the Department of Gynaecology and Obstetrics and Department of Urology, AIIMS, New Delhi.

### Semen analysis

Semen analysis was done twice at 2 wk interval. Samples were collected after minimum of 48 h and not longer than 7 d of sexual abstinence. The name of the patient, period of abstinence and time of collection were recorded on the form accompanying each semen analysis. Samples were collected in a private room near the laboratory and were delivered to the laboratory within 1 h after collection. The samples were obtained by masturbation and ejaculated into a clean, wide-mouthed glass or plastic container. The procedure for sample collection was explained to the patients and controls. Semen analysis was done as per WHO guidelines (1999).

### ROS detection by chemiluminescence assay in neat semen

The ROS production in 400  $\mu$ L of liquefied neat semen was measured after addition of 10  $\mu$ L of 5 mmol solution of luminol in DMSO (dimethylsulphoxide, Sigma Chemical Co.). A tube containing 10  $\mu$ L of 5 mmol luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, United States) solution in DMSO was used as a blank. Chemiluminescence was measured in duplicate for 10 min using the Berthold detection luminometer (United States). Sample analysis was done along with blank, positive control ( $H_2O_2$  + PBS + Luminol) and negative control (PBS + luminol). Results were expressed in RLU per second and per  $1 \times 10^6$  spermatozoa.

### 8-Isoprostane estimation

8-Isoprostane (8-IP) is a stable marker of oxidative stress which is indicative of lipid peroxidation. 8-IP levels are independent of dietary intake of lipids hence its level gives the absolute measurement of lipid peroxidation due to oxidative stress. 8-IP levels were estimated by ELISA. The quantification was done by Cayman's 8-IP EIA Kit. Protocol was followed as described by the manufacturer for the quantification.

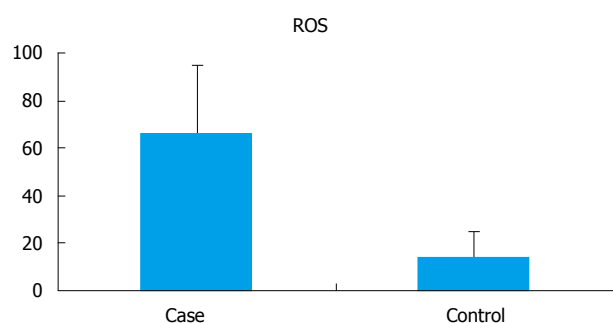
### Telomere length estimation

**Real time PCR:** Sperm telomere length was determined from the sperm DNA by a quantitative real-time PCR-based method. Briefly, the relative mean telomere length was determined by comparing the value from absolute quantification of telomere DNA with a single copy reference gene, 36B4 (T/S ratio). These two assays were carried out as separate reactions on separate plates maintaining the sample positions between the two plates. Amplification signals were quantified by the standard curve method using a DNA template series (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng/ $\mu$ L) on every plate. All randomized DNA samples (20 ng) and standard dilution was processed as triplicates on 96-well plates using Bio-Rad CFX 96 (Hercules, CA, United States). The purpose of the standard curve was

**Table 1** Comparison of semen parameters and age of infertile men and controls

Category	Age (mean $\pm$ SD, yr)	SC (mean $\pm$ SD)	FM (mean $\pm$ SD)	pH (mean $\pm$ SD)	Volume (mean $\pm$ SD, mL)
Infertile <i>n</i> = 112	31.71 $\pm$ 4.45	41.5 $\pm$ 36.6	44.25 $\pm$ 19.94	7.5 $\pm$ 0.55	3.04 $\pm$ 1.23
Controls <i>n</i> = 102	32.22 $\pm$ 4.0	70.66 $\pm$ 57.9	60.14 $\pm$ 18.33	7.61 $\pm$ 0.38	2.96 $\pm$ 1.07

SC: Sperm count ( $P = 0.0007$ ); FM: Forward motility ( $P < 0.0001$ ) (A + B grade in %).

**Figure 1** Seminal reactive oxygen species levels (RLU/s/million sperm) in cases and controls.

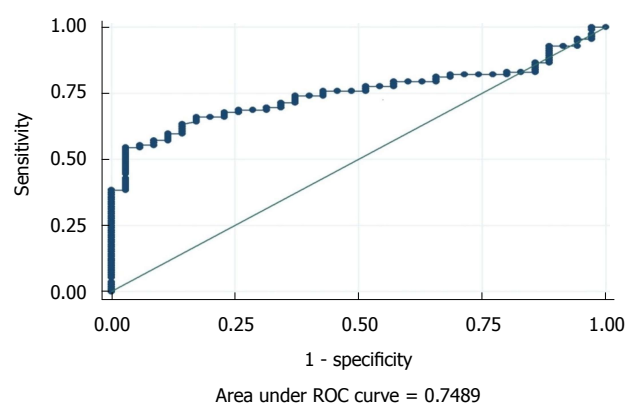
to assess and compensate for inter-plate variations in PCR efficiency. Amplification of the telomeric repeat region was expressed relative to amplification of 36B4, a single copy gene (SCG) encoding acidic ribosomal phosphoprotein located on chromosome 12. Real time kinetic quantitative PCR determines, for each sample well, the Ct, *i.e.*, the fractional cycle number at which the well's accumulating fluorescence crosses a set threshold that is several standard deviations above baseline fluorescence. A plot of Ct vs log (amount of input target DNA) is linear, allowing simple relative quantitation of unknowns in comparison to a standard curve derived from amplification, in the same plate, of serial dilutions of a known reference DNA sample. For this study, telomere (T) PCRs and SCG PCRs were always performed in separate 96-well plates.

## RESULTS

The sperm parameters, sperm count ( $P = 0.0007$ ) and forward motility ( $P < 0.0001$ ) were significantly lower in infertile men compared to controls and no significant difference in the seminal volume and pH was observed between infertile men and controls. Out of 112 cases, 70 men had normal semen parameters as per WHO 1999 guidelines (Table 1).

### ROS estimation

The seminal ROS levels in cases were significantly higher ( $66.61 \pm 28.32$  RLU/s/million sperm) than controls ( $14.04 \pm 10.67$  RLU/s/million sperm) ( $P < 0.0001$ ) (Figure 1). Receiver operating curve analysis was done to establish a cut off value for seminal ROS levels. The cut off obtained was 21.3 RLU/s/million sperm (Figure 2).

**Figure 2** Receiver operating characteristic curve for reactive oxygen species. Cut off = 21.3 RLU/s/million sperm, area under curve = 0.748, specificity = 77.14%. RLU: Relative light units; ROC: Receiver operating curve.

### 8-IP estimation

Seminal 8-IP levels were significantly ( $P < 0.001$ ) elevated in the cases ( $421.55 \pm 131.29$  pg/mL) as compared to controls ( $275.94 \pm 48.13$  pg/mL) (Figure 3).

### Telomere length estimation

The mean telomere length (T/S) in the infertile men ( $0.609 \pm 0.15$ ) was significantly lower ( $P < 0.0001$ ) when compared to controls ( $0.789 \pm 0.060$ ) (Figure 4). No significant difference in the average mean age of infertile men and control was observed. All analysis were adjusted for age. For the normalization of telomere length with age, cases and controls were divided into 2 groups according to age (group A: 18-30 years; group B: 31-45 years). There was no significant difference between the mean age of the cases compared to controls in the respective groups, but there was significant difference between telomere length in cases as compared to controls in both of these groups (group A cases:  $0.51 \pm 0.15$  vs controls:  $0.79 \pm 0.064$ ,  $P < 0.0001$ ; group B cases:  $0.50 \pm 0.11$  vs controls:  $0.77 \pm 0.05$ ,  $P < 0.0001$ ).

### Oxidative stress and telomere length

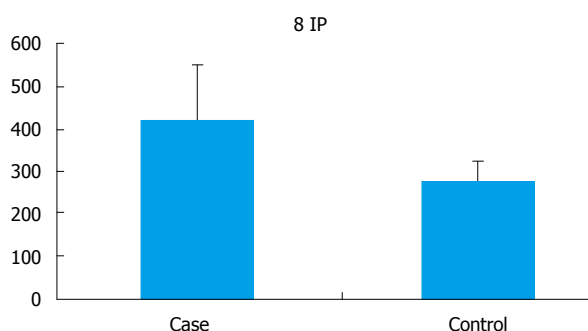
All the cases ( $n = 112$ ) were classified into three groups on the basis of seminal ROS levels. group I ( $n = 17$ ) had normal ROS levels (0-21.3 RLU/s/million sperm), group II ( $n = 40$ ) had mild ROS levels (21.3-35 RLU/s/million sperm) and group III ( $n = 55$ ) had severe ROS levels ( $> 35$  RLU/s/million sperm). The mean



**Table 2** Oxidative stress and telomere length

ROS (RLU/s/million sperm), (mean $\pm$ SD)	0-21.3 (normal) RLU/s/million sperm	21.3-35 (mild) RLU/s/million sperm	> 35 (severe) RLU/s/million sperm
Mean telomere length (mean $\pm$ SD)	0.663 $\pm$ 0.14	0.684 $\pm$ 0.12	0.595 $\pm$ 0.15
8-IP (pg/mL) (mean $\pm$ SD)	282.41 $\pm$ 61.97	440.54 $\pm$ 23.78	560.66 $\pm$ 43.82

ROS: Reactive oxygen species; RLU: Relative light units; 8-IP: 8-Isoprostane.

**Figure 3** 8-Isoprostane levels (pg/mL) in cases and controls.

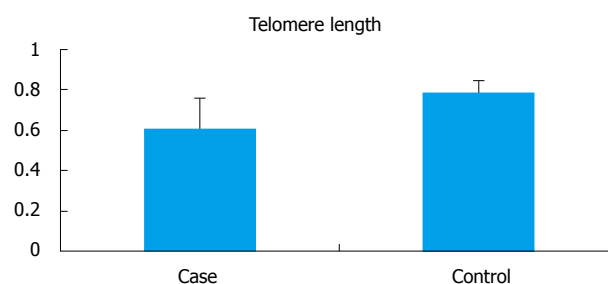
telomere length was analysed in these three groups and we observed a significant ( $P < 0.001$ ) shortening of telomere length in the group ( $0.595 \pm 0.15$ ) with ROS levels more than 35 RLU/s/million sperm as compared to other two groups (group I :  $0.663 \pm 0.14$  and group II :  $0.684 \pm 0.12$ ). Interestingly, it was observed that the mean telomere length was elevated in the patient group with mild oxidative stress ( $0.684 \pm 0.12$ ) as compared to the patient group with normal oxidative stress ( $0.663 \pm 0.14$ ) ( $P < 0.001$ ) (Table 2).

There were 23 (22.6%) controls that had ROS levels in the mild oxidative stress level range. When the mean telomere length in this group was compared with rest of the controls a similar pattern of increased telomere length was observed in the group of controls with mild oxidative stress ( $0.79 \pm 0.06$ ) as compared to the rest of the controls with normal oxidative stress levels ( $0.73 \pm 0.04$ ) ( $P = 0.01$ ).

8-IP levels also showed an increasing trend towards increasing ROS levels (Table 2). There was a negative correlation of sperm telomere length with ROS ( $r = -0.63$ ,  $P = 0.02$ ) and 8-IP ( $r = -0.33$ ,  $P = 0.03$ ) (Spearman's correlation). Also, telomere length was found to be negatively correlated with age ( $r = -0.4$ ,  $P = 0.035$ ).

## DISCUSSION

Telomeres and telomere associated proteins play an important role in the maintenance of genomic integrity in the eukaryotic cells. They also participate in the cellular DNA damage response pathways. Additionally, telomeres aid in the movement, localization, and anchoring of the chromosomes to the nuclear membrane. During cell division, telomeres mediate the pairing of homologous chromosomes, synapsis formation,

**Figure 4** Mean telomere length in cases and controls.

and homologous recombination<sup>[12]</sup>. Telomere length shortens with every cell division due to the inefficiency of the DNA polymerases to replicate the chromosome ends. Besides this mechanism of telomere shortening, oxidative stress is another important factor affecting the telomere dynamics in the nucleus. ROS induced telomere shortening may be due to direct oxidative injury to guanine bases in telomeric DNA.

Oxidative stress induces accelerated telomere shortening by the accumulation of oxidized DNA base products (8-OHdG) in the guanine rich telomeres which further recruit DNA damage response machinery that ultimately cause telomere attrition during the course of DNA damage repair. Accumulation of these base adducts cause single-strand breaks in the telomeric regions and these breaks are less efficiently repaired at telomeres than in the rest of the genome<sup>[13]</sup>. In this study we have observed elevated ROS levels in the sperm of infertile men as compared to the fertile controls. Limited studies exist exploring the effect of oxidative stress on the sperm telomere and all the studies so far direct towards a negative correlation between oxidative stress and telomere length.

It is now well documented that basal levels of ROS are essential for cell survival and subserve several physiological functions. It is evident from previous studies that severe oxidative stress leads to extensive damage to biomolecules and causes cell death<sup>[14]</sup> but moderate levels of ROS are beneficial to cellular homeostasis especially in cellular responses during pathological challenges in aging and aging-associated diseases<sup>[15]</sup>. ROS is generated as a part of normal aerobic cellular metabolism<sup>[16]</sup> and can be generated from various metabolic pathways<sup>[17]</sup>, including electron transport during oxidative phosphorylation in the mitochondria<sup>[18]</sup>, dihydrolipoamide dehydrogenase in the  $\alpha$ -keto acid dehydrogenase complexes<sup>[19]</sup>, NADPH

oxidase and, xanthine oxidase and, monoamine oxidase, and cytochrome P450 proteins<sup>[20]</sup>.

Although basal levels of ROS are pivotal for redox signalling<sup>[21]</sup> and cell survival, high levels of ROS would be lethal to normal cellular processes. High levels of oxidative stress are believed to contribute a major part in cellular aging and several aging-related diseases. A moderate level of oxidative stress is needed for various cellular responses beneficial for cell survival<sup>[22]</sup>.

As ROS are transient molecules with very short half lives they execute beneficial effects by activating downstream molecules. These downstream molecules include oxidative products such as lipid peroxidation by-products and protein oxidation adducts. These modifications include disulfide formation, S-glutathionylation, S-sulfenation, and S-nitrosylation<sup>[23]</sup>. These modifications induced by ROS regulate protein function and thus protect them from severe stress conditions. These effectors of moderate oxidative stress can actually execute the ultimate function of positive oxidative stress by redox signaling and activation of transcription factors<sup>[24]</sup>. Since oxidative stress plays a major role in chronic inflammatory diseases, telomere attrition may be involved in the pathophysiology of these diseases. Several studies have linked telomere shortening to various chronic metabolic and inflammatory diseases such as atherosclerosis, diabetes, inflammatory bowel disease, and chronic obstructive pulmonary disease conditions that are all characterized by the presence of systemic oxidative stress<sup>[13]</sup>. However, the exact underlying mechanisms of telomere shortening under conditions of chronic oxidative stress have not been elucidated. In the present study we found that the infertile group with mild oxidative stress (21.3-35) had longer telomeres as compared to the group with normal free radical levels and group with severe oxidative stress emphasizing that the slightly elevated free radical levels are beneficial for telomere length maintenance<sup>[25]</sup>. The mean telomere length decreased in the patients as compared to the controls but it was increased in the patient group that had mild oxidative stress. The result of our study can be explained by findings of Wang *et al.*<sup>[26]</sup>. It was documented that, when few base lesions affect telomeric DNA repeats, they reduce binding of proteins in telomeres which liberate the negative regulation of telomere binding proteins on telomerase and consequently increase telomerase-dependent telomere repeat additions such that telomere lengthening occurs. However severe oxidative stress causes complete uncapping of telomeric DNA with oxidized bases and consequently activate DNA damage response and results in telomere shortening. As already documented, physiological levels of free radical maintain homeostasis and positive oxidative stress can be induced by non lethal free radicals<sup>[27]</sup>. There are also positive effects of physiological level of ROS in *in vivo* conditions and cellular environment also plays an important role in regulating telomere length and telomerase activity<sup>[28]</sup>.

Recently, it has been shown that telomeric regions

are favoured targets of a persistent DNA damage response induced by genotoxic and oxidative stress, both *in vitro* and *in vivo*<sup>[29]</sup>. Oxidative stress induces single-strand breaks both directly and indirectly. These are less efficiently repaired in telomeric DNA as compared to genomic DNA<sup>[30]</sup>. Kawanishi *et al.*<sup>[31]</sup> also stated that, formation of 8-OHdG at the GGG triplet in telomere sequence induced by oxidative stress could accelerate telomere shortening. Accumulation of oxidized bases beyond a certain level in telomeres may severely deplete telomere binding proteins in telomeres and result in telomere uncapping. Uncapped telomeres can become targets for ATM or ATR kinases and nucleolytic degradation that eventually cause telomere shortening and cell cycle arrest<sup>[26]</sup>. Therefore, very high oxidative stress levels are detrimental to telomere length maintenance as is evident from the study. Infertility may thus be accelerated with oxidative stress, DNA damage and shorter telomeres. However mild oxidative stress is beneficial for telomere length and results in longer telomeres and thus may aid in maintenance of genomic integrity. Thus severe oxidative stress and resultant shorter telomeres may impair spermatogenesis and result in hypospermatogenesis and may manifest as oligozoospermia and later as azoospermia however mild oxidative stress aids in maintenance of telomeres and thus aid in mitotic division of germ cells. Thus one should not indiscriminately take antioxidants and levels of seminal free radical levels should be monitored regularly when one is on antioxidants. Recent studies from our laboratory have documented the beneficial effect of yoga and meditation in reducing oxidative stress and upregulating activity of telomerase enzyme. Thus we believe that infertility is actually accelerated testicular aging characterised by oxidative stress, DNA damage and shorter telomeres and it could be reversed by adopting yoga and meditation into daily lifestyle.

With available evidence that physiological levels of free radical is important for various cellular, physiological and biochemical mechanisms including regulation of gene expression and cell signalling a delicate balance for free radical levels needs to be established to maximize the beneficial physiological effects of free radicals and minimize the detrimental effects of supra physiological levels.

## COMMENTS

### Background

Infertility is a complex life style disorder. Oxidative damage to sperm DNA is one of the major causes of infertility especially in men with normal sperm parameters. Recent studies have shown that loss of sperm DNA integrity, hypermutability of mitochondrial genome, altered gene expression, short telomere length and environmental/lifestyle factors like smoking, psychological stress are the key players in male infertility. DNA damage is chiefly oxidative and one of the leading causes of defective sperm function. Simple lifestyle modifications can significantly improve DNA integrity.

### Research frontiers

Various studies have explored the role of oxidative stress and DNA damage in male infertility but there are few studies to understand the role of sperm

telomeres and its length in the aetiology of male infertility. Numerous studies have addressed the detailed description of telomere in various diseases but the telomere length in the sperm and their correlation with seminal oxidative stress has not been intensely investigated. There are few studies on the role of telomere in reproduction and since oxidative stress has such a marked effect on telomere phenotype, it is important to evaluate the effect of oxidative stress (normal, mild, severe) on telomere length. It is important to establish cut off levels of free radicals which are beneficial to telomeres and cause telomere lengthening as compared to very high reactive oxygen species (ROS) levels which induce DNA damage response in telomeres and cause telomere shortening.

### Innovations and breakthroughs

In this study the authors found longer telomere length in cases with mild oxidative stress levels [21.3-35 relative light units (RLU)/s/million sperm] as compared to cases with normal ROS levels (0-21.3 RLU/s/million sperm). Thus it would be more valuable to establish cut off free radical levels which are beneficial/detrimental to telomere length maintenance. The authors have investigated the role of oxidative stress in sperm telomere length maintenance in idiopathic male infertility.

### Applications

Thus severe oxidative stress and resultant shorter telomeres may impair spermatogenesis and result in hypospermatogenesis and may manifest as oligozoospermia and later as azoospermia however mild oxidative stress aids in maintenance of telomeres and thus aid in mitotic division of germ cells. One should not indiscriminately take antioxidants and levels of seminal free radical levels should be monitored regularly when one is on antioxidants.

### Peer-review

This is a quite well designed study which is worth to be published in scientific journal. Language is communicative, manuscript is concise, methods are well chosen, results transparent and discussion sufficient.

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Retrospective Study

# Footwear modification following hallux valgus surgery: The all-or-none phenomenon

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## Abstract

**AIM:** To define footwear outcomes following hallux valgus surgery, focusing on patient return to comfortable and heeled footwear and patterns of post-operative footwear selection.

**METHODS:** Surgical intervention is indicated for symptomatic cases of hallux valgus unresponsive to conservative methods, with favourable reported outcomes. The return to various types of footwear post-operatively is reflective of the degree of correction achieved, and corresponds to patient satisfaction. Patients are expected to return to comfortable footwear post-operatively without significant residual symptoms. Many female patients will additionally attempt to return to high-heeled, narrow toe box shoes. However, minimal evidence exists to guide their expectations. Sixty-five female hallux valgus patients that had undergone primary surgery between 2011 and 2013 were retrospectively identified using our hospital surgical database. Patients were reviewed using a footwear-specific outcome questionnaire at a mean 18.5 mo follow-up.

**RESULTS:** Eighty-six percent of patients were able to return to comfortable footwear post-operatively with minimal discomfort. Of those intending to resume wearing heeled footwear, 62% were able to do so, with 77% of these patients wearing these as or more frequently than pre-operatively. No significant difference was observed between pre- and post-operative heel size. Mean time to return to heeled footwear was 21.4

wk post-operation. Cosmetic outcomes were very high and did not adversely impact footwear selection.

**CONCLUSION:** We report high rates of return to both comfortable and heeled shoes in female patients following primary hallux valgus surgery. We observed an “all-or-none phenomenon” where patients rejected a return to heeled footwear unless able to tolerate them at the same frequency and heel size as pre-operatively. A minority of patients were unable to return to comfortable footwear post-operatively, which had adverse ramifications on their quality-of-life. We recommend that the importance of managing patient expectations through appropriate pre-operative counselling be emphasized in forefoot surgery.

**Key words:** Hallux valgus; Bunion; Footwear; High-heels; Scarf osteotomy; Lapidus procedure

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**Core tip:** Footwear outcomes following primary hallux valgus surgery are favourable, with the majority of patients returning to comfortable footwear post-operatively with minimal to no discomfort. An additional cohort of female patients will attempt to return to heeled footwear. Nearly two-thirds of these patients tolerated heeled footwear post-operatively, the majority of these at the same heel size and frequency of use as pre-operative levels. Appropriate pre-operative counselling is imperative to achieving high patient satisfaction with footwear outcomes following hallux valgus surgery.

Robinson C, Bhosale A, Pillai A. Footwear modification following hallux valgus surgery: The all-or-none phenomenon. *World J Methodol* 2016; 6(2): 171-180 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i2/171.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i2.171>

## INTRODUCTION

Hallux valgus deformities (Figure 1) are one of the most common reported foot complaints, affecting greater than 35% of individuals 65+<sup>[1,2]</sup>. The prevalence is significantly higher in females and increases with advancing age<sup>[1,2]</sup>. The pathophysiology remains poorly defined, but current hypotheses implicate an interplay between genetic predisposition, initiating, and aggravating factors<sup>[2-4]</sup>. In females, the use of high-heeled narrow toe-box (HHNT) footwear (Figure 2), common in fashionable styles, has been identified as both an initiating and aggravating factor<sup>[3-5]</sup>. Patients reporting significant pain or functional impairment unresponsive to conservative methods (footwear modification, orthotics and non-steroidal anti-inflammatories) should be considered for joint-preserving surgical intervention (e.g., scarf ± akin osteotomy)<sup>[6-10]</sup>. The safety and efficacy of these

procedures in the treatment of hallux valgus deformity is well described<sup>[6,8-13]</sup>.

The return to comfortable footwear (e.g., trainers, flats, boots) is a fundamental aspect of post-operative rehabilitation and is closely related to overall patient satisfaction<sup>[8,11,14-16]</sup>. Patients are typically counselled to anticipate a period of post-operative swelling and discomfort proportional to the required degree of correction and the amount of soft tissue disruption. Previous reports suggest that patients should expect to return to comfortable footwear between 6 wk and 6 mo post-operatively, depending on operative and patient factors<sup>[6,7,11,17]</sup>.

However, the evidence documenting footwear outcomes following hallux valgus surgery is limited. No studies have attempted comparison between age cohorts or procedure type. Minimal information exists on the rates of return and patterns of HHNT footwear use post-operatively. Many patients seek pre-operative information on the probability and timescales in returning to HHNT footwear. The provision of realistic, evidence-based pre-operative counselling is critical to adequately managing their expectations. The aim of our study is to determine the impact hallux valgus surgery has on patient footwear and assess the ability of female patients to return to comfortable and HHNT shoes post-operatively.

Our scientific hypothesis is that the vast majority of both working and retirement age patients will return to comfortable footwear with minimal residual symptoms. Additionally, a small cohort of patients will successfully return to wearing HHNT at pre-operative levels. The rates of return to HHNT footwear will be higher in working age patients, as will patient-reported rates of significant footwear restriction. Patients undergoing scarf procedure ± akin (alone) will have the lowest rates of post-operative footwear restriction.

## MATERIALS AND METHODS

Following approval from our institutional review board, we performed a retrospective review of our surgical database to identify female patients that had undergone corrective surgery for hallux valgus between January 1<sup>st</sup>, 2011 and June 28<sup>th</sup>, 2013. All patients aged 18+ operated on at our institution between these dates were included for participation in our study.

Sixty-five patients were reviewed at a mean time to follow-up of 18.5 mo (range: 6.9 to 35.9 mo). Mean patient age for our study population was 59.3 years. It is recognized that footwear selection and functional capacity varies considerably by patient age. In an attempt to identify differences between age cohorts, we segregated participants into two groups: Cohort A (18-64) - 42 individuals and cohort B (65+) - 23 individuals. While we recognized that overlap would exist in footwear selection between these cohorts, it was felt to be sufficient for rudimentary comparative analysis. In 2012, the average effective retirement age

**Table 1 Footwear/cosmetic outcomes following hallux valgus surgery: Patient questionnaire**

Patient study ID:  
 Date of birth:  
 Were you given information pre-operatively on what to expect regarding footwear following surgery?  
 Yes/No - (Comments):  
 What impact has foot surgery had on your choice of footwear? Are you still restricted in your footwear?  
 Able to tolerate any footwear  
 Able to tolerate only comfortable footwear  
 Unable to tolerate normal, comfortable footwear  
 (Comments):  
 Did you wear high-heeled footwear ( $\geq 3$ cm) before your operation? Have you been able to wear these since? If so, when were you able to resume?  
 Pre: Routine/occasional/never  
 Post: Routine/occasional/never  
 (1) Heel size?  
 (2) Return time (in weeks):  
 Did you desire to return to heeled footwear following your operation?  
 Yes / No - (Comments):  
 How would you rank the appearance of your feet pre-operatively?  
 1            2            3            4            5            6            7            8            9            10  
 (Comments):  
 How would you rank the appearance of your feet post-operatively?  
 1            2            3            4            5            6            7            8            9            10  
 (Comments):  
 Does the post-operative appearance (e.g., any scars or bumps) discourage you from wearing any types of footwear?  
 Yes/No - (Comments):



**Figure 1 Hallux valgus (pre-/post-op).** "Hallux valgus und Krallenzehe II" and "Dieselbe Patientin wie oben nach Austin-Korrektur (I) und PIP-Arthrodese (II)", January 1 2013, Archiv Dr. med, Angela Simon, Malchin, Licensed under CC BY-SA 3.0. Available from: URL: [https://upload.wikimedia.org/wikipedia/commons/7/7d/Hallux\\_valgus\\_und\\_Krallenzehe\\_II\\_\(pr%C3%A4op.\).jpg](https://upload.wikimedia.org/wikipedia/commons/7/7d/Hallux_valgus_und_Krallenzehe_II_(pr%C3%A4op.).jpg) and Available from: URL: [https://de.wikipedia.org/wiki/Hallux\\_valgus#/media/File:Korrektur\\_I.\\_und\\_II.\\_Zehe\\_\(postop.\\_Nach\\_OP\\_nach\\_Austin\\_und\\_PIP-II-Arthrodese\).jpg](https://de.wikipedia.org/wiki/Hallux_valgus#/media/File:Korrektur_I._und_II._Zehe_(postop._Nach_OP_nach_Austin_und_PIP-II-Arthrodese).jpg)

for United Kingdom females was reported to be 63.1 years by the Organisation for Economic Co-operation and Development<sup>[18]</sup>. For the purposes of our study, 65 years was set as the cut-off between working and retired age cohorts. We additionally segregated patients by operation type, to assess its impact on footwear outcomes. Of the 65 patients, 20 underwent scarf  $\pm$  akin osteotomy, 15 underwent scarf  $\pm$  akin + additional joint fusion/correction and the remaining 30 were described to have undergone Lapidus tarsal-metatarsal (TMT) fusion.

A footwear-specific outcomes questionnaire was designed for the purposes of this study, in order to assess the impact that surgery had on the footwear selection of our patients (Table 1). This questionnaire



**Figure 2 High-heeled narrow toe box footwear.**

was designed using elements specific to patient footwear from three validated functional scoring systems: The American Orthopaedic Foot and Ankle Society (AOFAS) Hallux/MTP/IP scale, the American Academy of Orthopaedic Surgeons (AAOS) Foot and Ankle Questionnaire, and the Manchester-Oxford Foot Questionnaire. We defined comfortable footwear as: Normal-fitting, non-prescription shoes with a heel size  $< 3$  cm and a wide toe-box. The most common types of shoes included were trainers, flats, boots, and casual shoes. HHNT footwear was defined as: Tight-fitting shoes with a heel size  $\leq 3$  cm  $\pm$  a narrow toe-box. Types included were pumps, wedges, platforms, slingbacks and stilettos.

## RESULTS

Patient-reported cosmetic appearance of the feet improved significantly following surgery, with 61 patients (93.8%) describing an improvement (Figure 3). No significant differences in cosmetic outcomes were

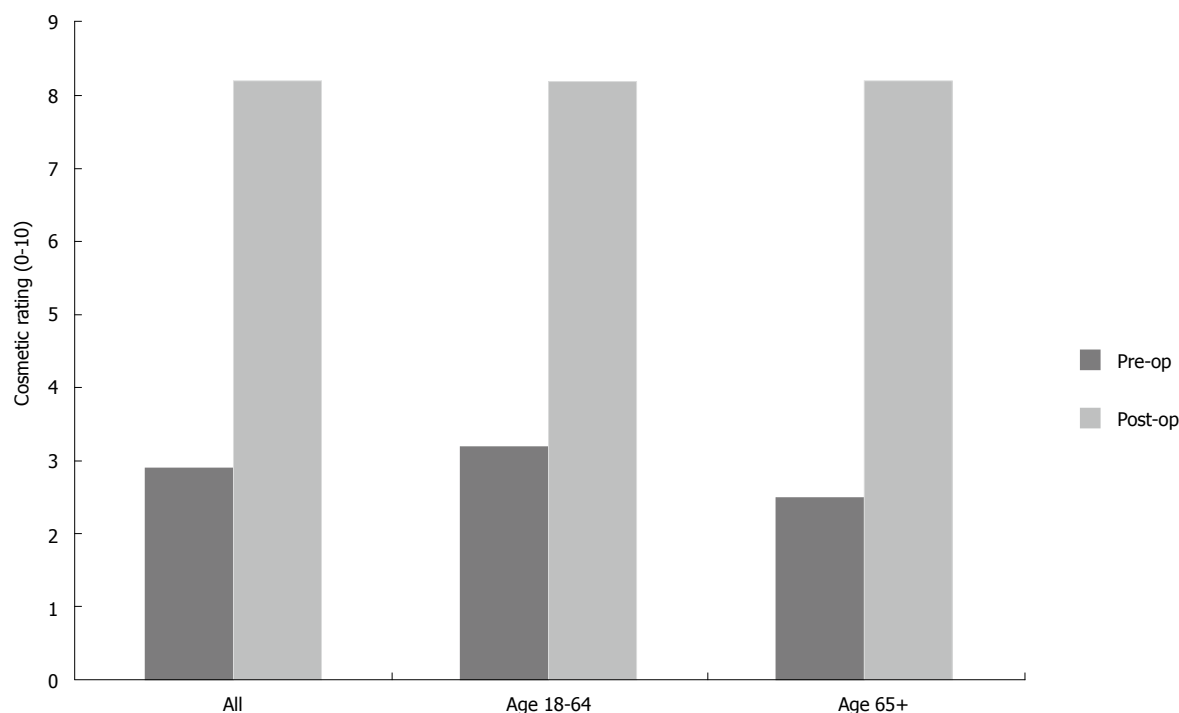


Figure 3 Cosmetic rating following hallux valgus surgery (by age).

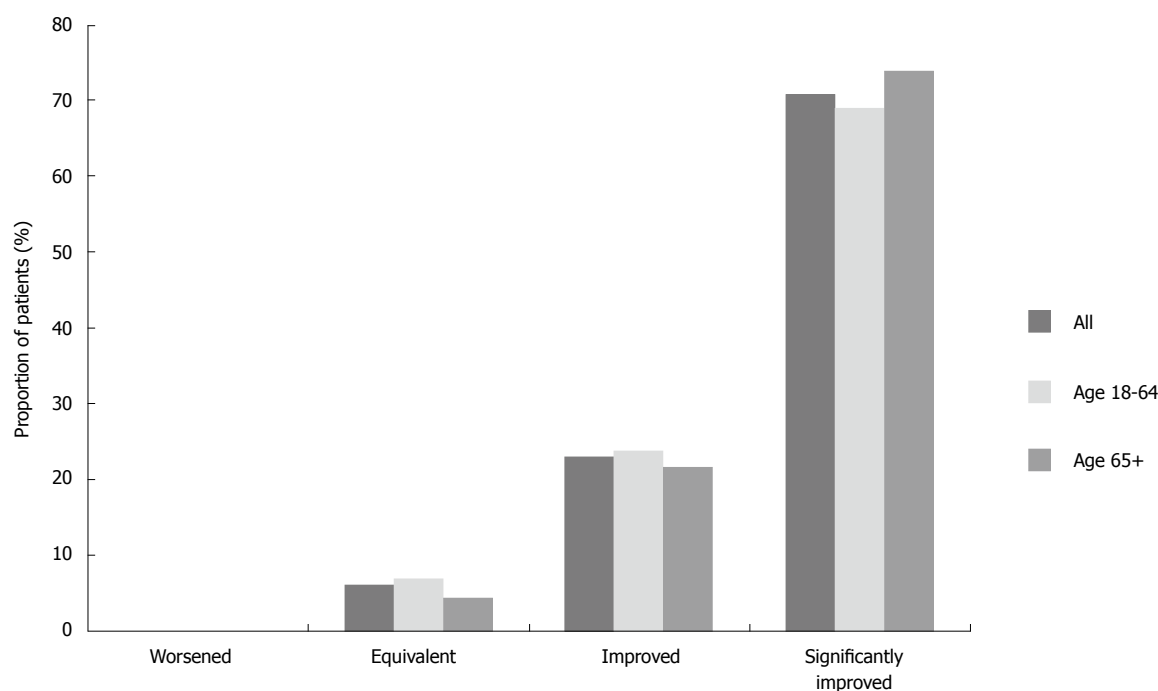


Figure 4 Degree of post-operative cosmetic improvement (by age).

found between cohort A (18-64) and cohort B (65+) individuals. No patients reported a worse post-operative cosmetic score. Further, 46 patients (70.8%) reported that their cosmetic appearance increased by  $\geq 5$  points on a 10-point global rating scale (Figure 4). Fifty-nine patients (90.7%) reported that the post-operative appearance of their feet did not adversely impact their footwear selection.

At follow-up, 38 patients (58.5%) reported minimal restriction (indicating the ability to wear comfortable shoes but difficulty with HHNT ones) (Figure 5A). A further 18 patients (27.7%) described being unrestricted in their footwear (indicating the ability to wear both comfortable as well as HHNT shoes without residual discomfort). Significant restriction (indicating the inability to wear normal, comfortable shoes without



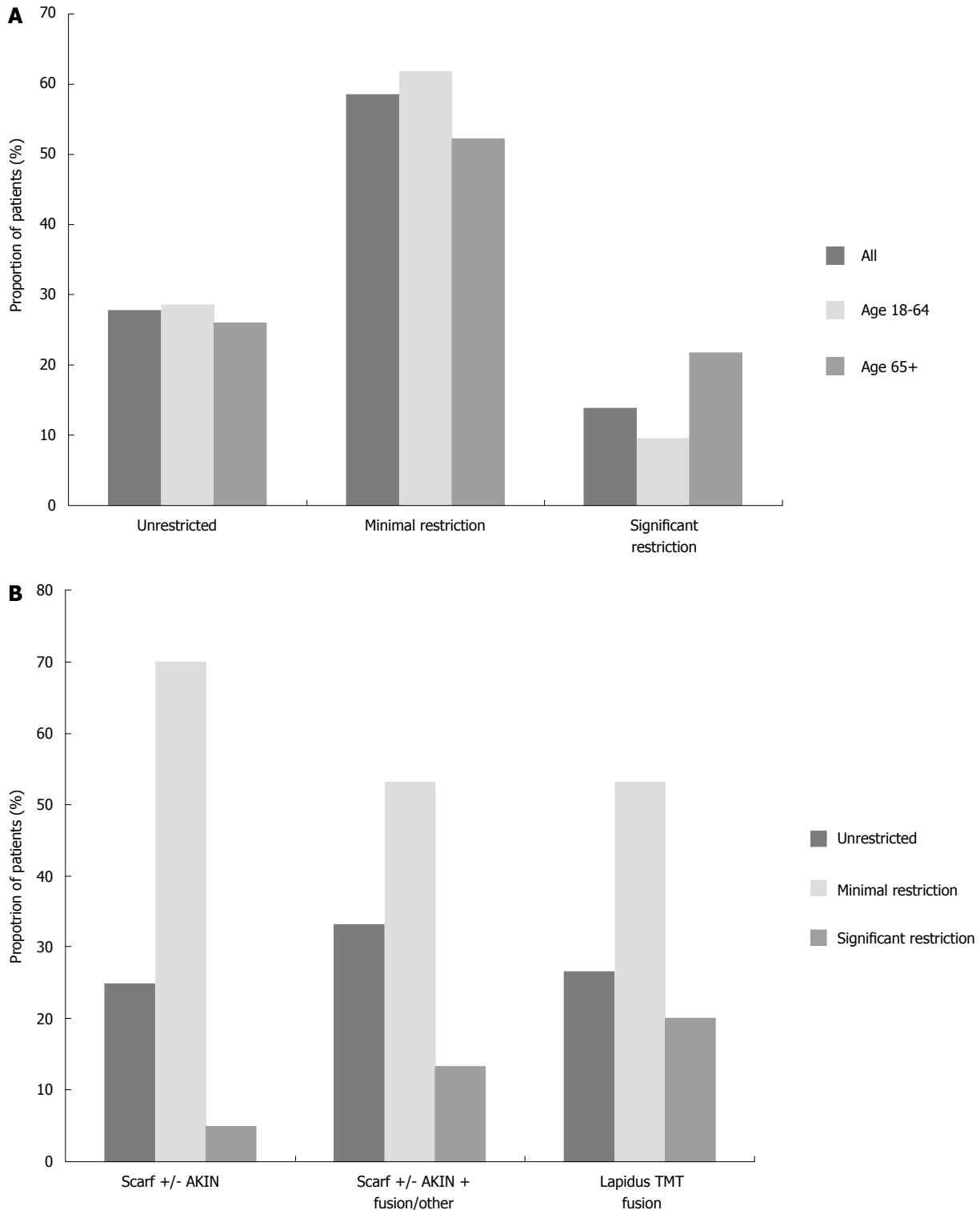


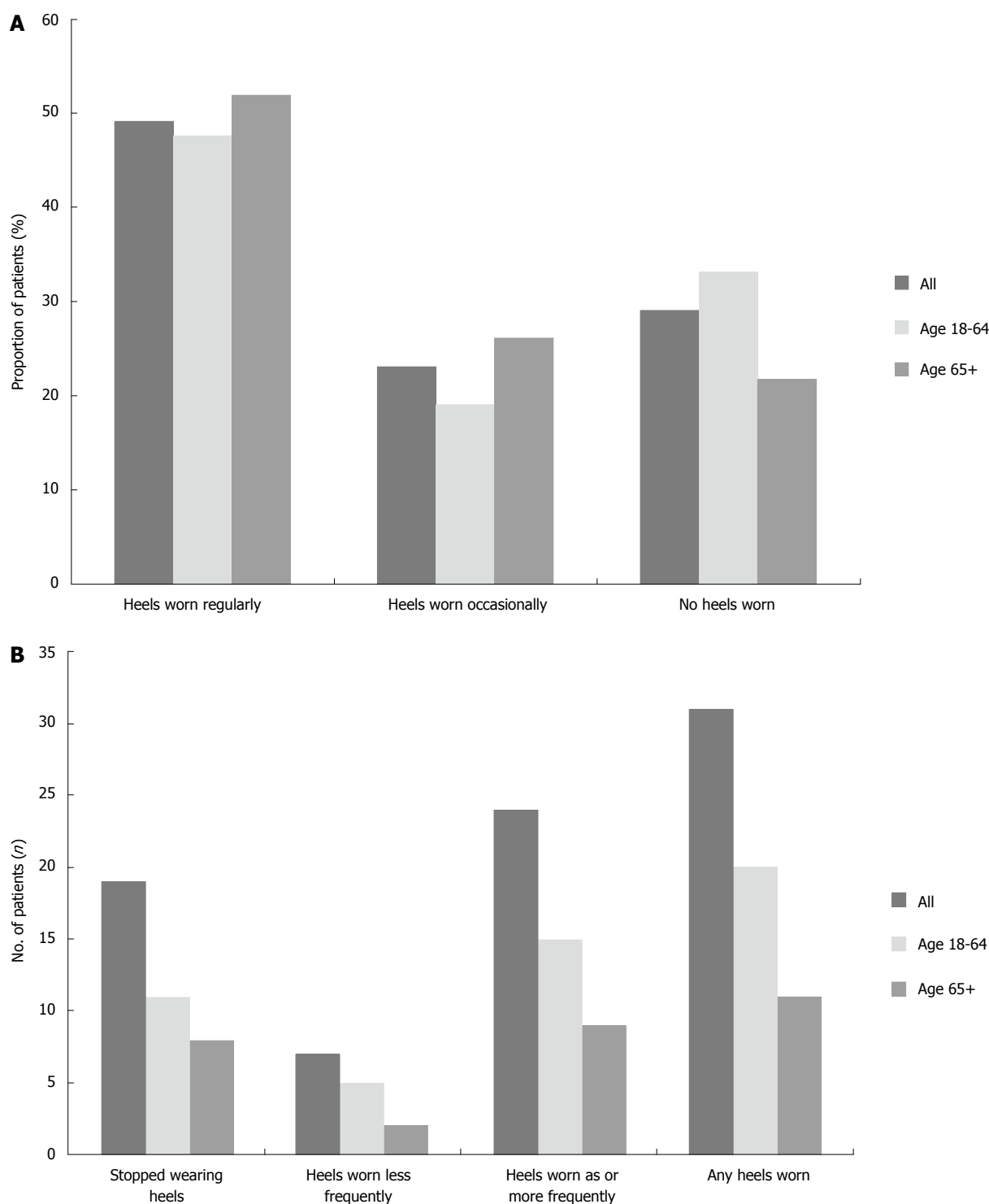
Figure 5 Footwear restriction following hallux valgus surgery (A: by age) and (B: by procedure).

significant discomfort) was reported by 9 patients (13.9%). Those in retirement age cohort were twice as likely to report significant restriction in their footwear.

When compared by operation type, the lowest rates of significant restriction were observed following scarf  $\pm$  akin operation alone - 1 patient (5%) (Figure 5B). Those that underwent scarf  $\pm$  akin + additional toe fusion/correction and those that underwent Lapidus

TMT fusion had higher rates of significant restriction - 2 patients (13.3%) and 6 patients (20%), respectively.

Only 19 patients (29.2%) reported minimal or no use of HHNT shoes directly before their surgery. This was related to discomfort over the medial bony prominence and/or metatarsalgia (Figure 6A). Interestingly, working age patients were less likely to have used heeled footwear pre-operatively (28 patients,



**Figure 6** Heel use. A: Pre-operative heel use (by age); B: Post-operative heel use (by age).

66.7%) than their retirement age counterparts (18 patients, 78.3%).

Fifty of our 65 participants (76.9%) expressed the desire to return to HHNT footwear post-operatively. Of these 50 patients, 31 (62%) reported that they had resumed use following the operation (Figure 6B). Additionally, 24 of these patients (77.4%) described use at equal or greater frequency than they had pre-operatively. Of the 19 patients that did not wear HHNT

footwear pre-operatively, 4 (21.1%) began wearing them following their surgery. There was no significant difference in likelihood of patients returning to HHNT footwear post-operatively between working and retirement age cohorts.

The mean time taken to return to HHNT footwear was 21.4 wk (range: 6 to 52) (Figure 7). Our retirement age cohort, however, took significantly less time to do so; 16.4 wk vs 24.1 wk (working age). Heel size

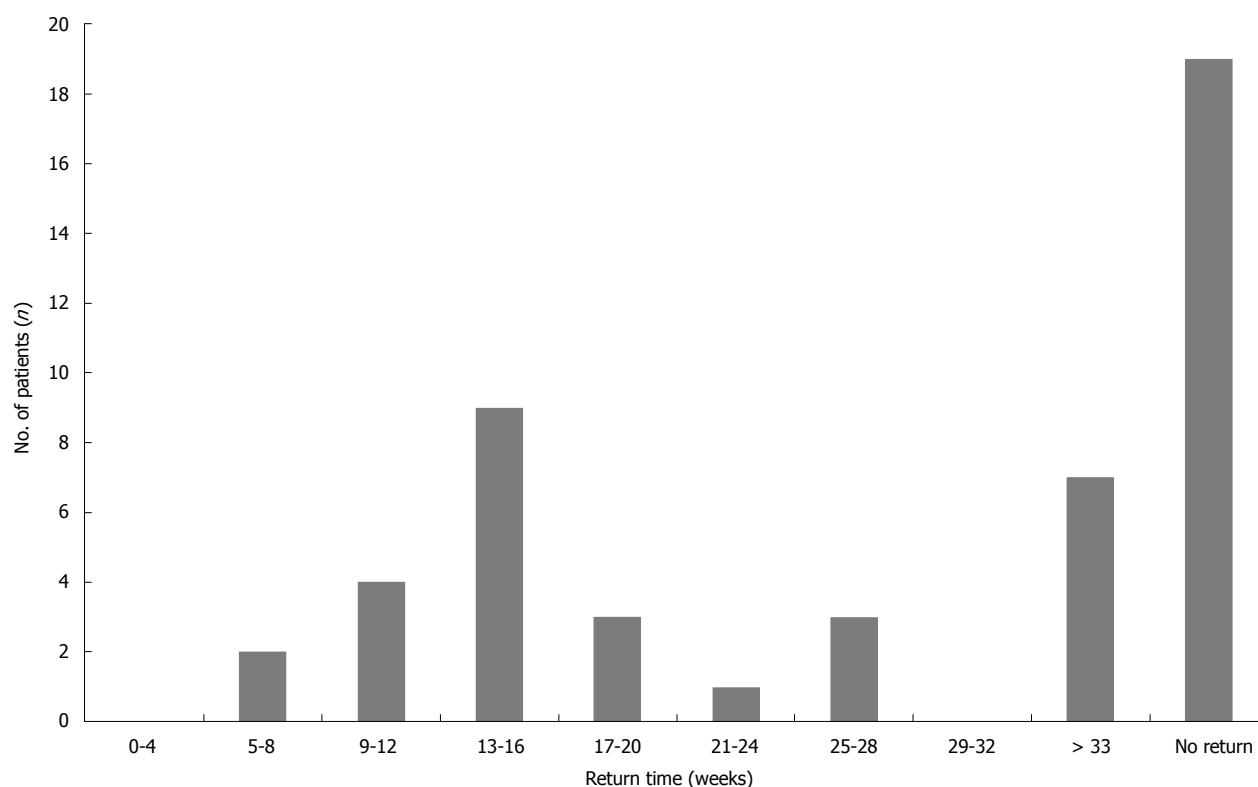


Figure 7 Return to heeled footwear.

remained constant following surgery and no significant difference was found pre- or post-operatively between age cohorts (Figure 8).

## DISCUSSION

Footwear outcomes following hallux valgus surgery remains disputed within the literature. Adam *et al.*<sup>[11]</sup> (2011) found an increase in AOFAS score from 61.5 pre-op to 90.3 post-op and reported that “patients returned to wearing dress shoes in 3.4 mo”. Dawson *et al.*<sup>[15]</sup> (2007) reported that more than half of their patients expressed the importance of the ability to wear fashionable shoes with a heel > 3 cm post-operatively. At 12-mo follow-up, 62.9% were very pleased with the appearance of their foot, 72.5% were either very or fairly pleased with the range of shoes they could tolerate and 87.1% reported reduced foot pain. They found that the most critical variable to post-operative satisfaction in women was “the appearance of their foot” and that this was closely related to the “range of shoes that they were able to wear”. Thordarson *et al.*<sup>[17]</sup> (2005) described that “postoperatively, most patients resumed fashionable or conventional shoes without an insert”. However, appraisal of their results showed that, despite overall improvement in the AAOS Foot and Ankle Core score, the standardized Shoe Comfort score remained 58.7 out of 100 at 24 mo follow-up.

Mann and Pfeffinger<sup>[19]</sup> (1991) were one of the first to report adverse footwear outcomes. They found that only 63.8% of patients were able to tolerate unrestricted

footwear post-operatively. Torkki *et al.*<sup>[9]</sup> (2001) described moderate to severe footwear restriction in 75% of their surgery cohort at 6-mo follow-up. Sixty-four point six percent continued to report these levels of restriction at 12-mo follow-up. Coetzee<sup>[16]</sup> (2003) reported unrestricted use of footwear at 12-mo follow-up in only 37% of their patients. Forty-two percent were restricted to trainers or comfortable shoes, many still reliant on orthotic inserts. Finally, Spruce *et al.*<sup>[20]</sup> (2011) found that only 51% of participants achieved “minimally-important difference” in shoe change following surgery.

Tai *et al.*<sup>[14]</sup> (2008) specifically focused on pre-operative patient expectations in hallux valgus surgery. They reported the ability to resume wearing normal shoes and dress shoes were ranked as the 3<sup>rd</sup> and 5<sup>th</sup> most important factors to patients, respectively, behind: (1) improved walking; and (2) reduced pain. Return to dress shoes was of greater importance to younger patients, whereas the return to comfortable shoes was ranked higher in those over age 40.

Our study demonstrated excellent post-operative cosmetic results (Figure 4). While cosmesis should not be regarded as an operative indication, improvement following surgery has been associated with high patient satisfaction and should be regarded as a desirable outcome<sup>[9,13,14]</sup>.

We found that the majority of our patients were able to return to these comfortable shoes without residual discomfort, demonstrating satisfactory functional outcomes. Additionally, nearly one-third of our patients reported the ability to tolerate the unrestricted use of

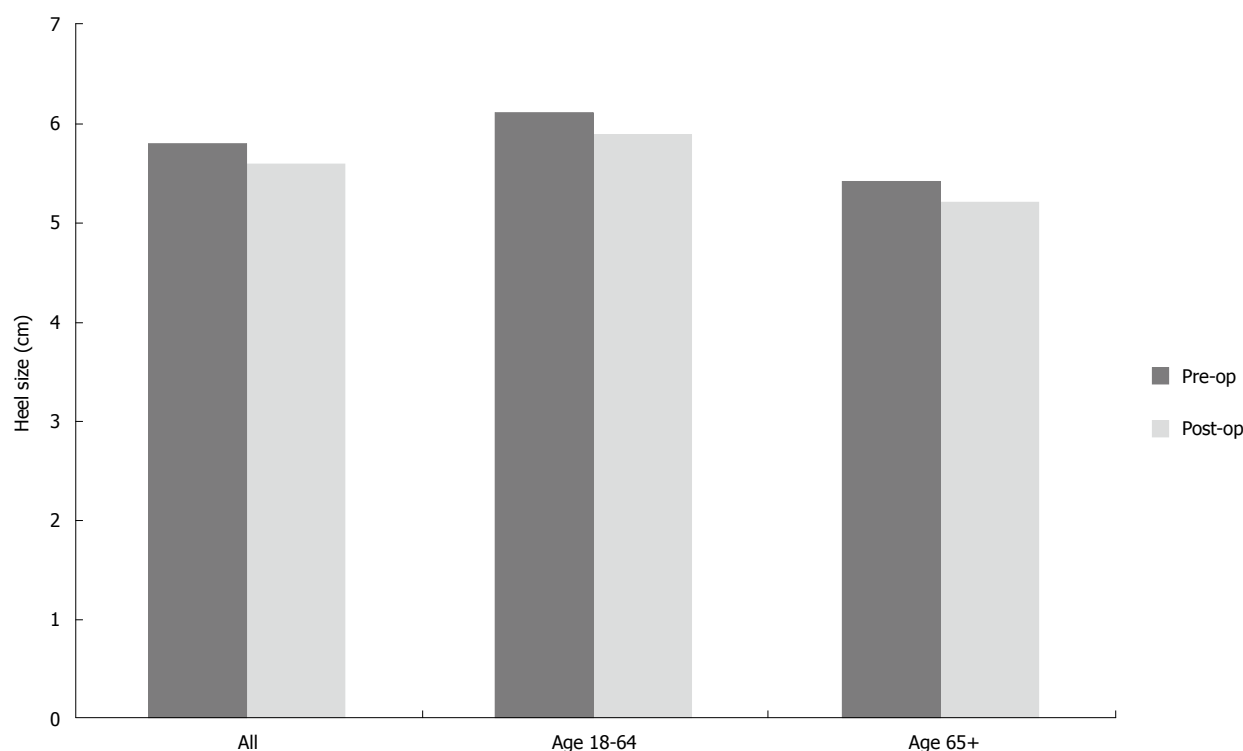


Figure 8 Heel size (by age).

both comfortable and HHNT footwear, confirming the high functional capacity recovered by some patients (Figure 5A). However, 13.9% of study population reported marked difficulty wearing comfortable footwear at follow-up. These patients generally described themselves as limited to custom-built orthotic footwear or loose-fitting slippers. As anticipated, patients that had undergone additional correction procedures or fusions reported greater restriction in their post-operative footwear. Four times as many patients reported significant restriction following a Lapidus TMT fusion than following a joint-preserving scarf  $\pm$  akin osteotomy alone (Figure 5B). Greater restriction in these patients is likely to correspond to increased soft tissue disruption and reduced functional mobility following surgery.

For working age patients, the impact of footwear modification on quality-of-life measures must be accounted for in pre-operative patient education. For the minority that remained significantly restricted in their footwear at follow-up, the professional and personal implications of such a limitation should not be understated. No patients in our study described an inability to return to work. However, many reported the need to modify their work environments and responsibilities for up to a year post-operatively. These observations highlight the clinical need for further investigation quantifying the impact of occupation on footwear outcomes.

In our series, the majority of patients intending to return to heeled footwear following surgery were able to do so. Further, 77.4% of these patients had increased or maintained the frequency of heel use from

pre-operative levels (Figure 6B). Heel size was not found to have decreased significantly following surgery (Figure 8). These findings are suggestive of an “all-or-none phenomenon” in footwear modification following hallux valgus surgery, indicating that female patients are unlikely to resume wearing any heeled footwear until symptom resolution enables them to wear heels of the same height and frequency as they could pre-operatively.

The simplest explanation for these findings is that they are reflective of wardrobe choice, where patients choose to wear what is already available to them and are reluctant to change their footwear selection for financial or cosmetic reasons. While this undeniably plays a role in post-operative footwear modification, we suggest that the phenomenon demonstrates a more complex interaction between psychological denial of the disease state and dissatisfaction with less than complete functional recovery. Perceived fashion and societal pressures for the patient, while not always congruent with surgical outcome measures, are also likely to contribute to this finding.

As such, we suggest that our findings may misrepresent genuine functional limitation. A degree of the restriction reported is likely self-imposed by patients. This likely relates to the reluctance towards making a partial return to HHNT footwear or shifts in patient preference towards more comfortable styles. Some participants express reluctance to resume wearing heeled footwear over concerns about the possibility of disease recurrence. Many patients are aware of the association between HHNT footwear and hallux valgus



deformity and this may contribute to avoidance of these footwear types as a prophylactic measure to reduce their risk of recurrence.

Following hallux valgus surgery, patients should be recommended to modify their footwear based on the residual symptoms they experience. Many patients, however, will desire to return to HHNT footwear post-operatively. There is no published evidence, at present, that the continued use of this footwear increases the risk of disease recurrence. However, the risk HHNT footwear use confers towards the development of primary hallux valgus deformity may apply equally to disease recurrence<sup>[4,21,22]</sup>. Finally, increasing heel size and smaller toe-boxes are likely to result in greater post-operative forefoot discomfort. It is therefore not advisable to recommend the frequent or prolonged use of HHNT footwear following surgery. It must, however, be accepted that for professional or personal reasons the majority of patients are likely to attempt to resume wearing HHNT footwear post-operatively. We recommend that the best approach is therefore to provide accurate and unbiased information about the likelihood of them being able to do so, as well as the potential risks involved.

Managing patient expectation and pre-operative education plays a pivotal role in outcomes following surgery to the foot and ankle. In practice, insufficient pre-operative information is likely to foster unrealistic expectations about post-operative footwear outcomes. In those patients requesting surgical intervention that are still able to achieve pain relief by footwear modification from HHNT to comfortable options, the primary focus should be to manage their symptoms non-operatively. This can be justified through reinforcement of the available evidence on post-operative footwear restriction and the operative risks involved. The results of our study should serve to enhance the pre-operative clinical advice provided to patients regarding their return to comfortable and HHNT footwear following primary hallux valgus surgery.

There are a number of limitations in our study design that affect the validity and reproducibility of the results. As a retrospective case series, there is significant potential for recall bias in patient-reported symptoms and footwear use. By excluding patients  $\geq 3$ -year post-operation we limited maximum follow-up time in an attempt to minimize the risk of bias. A further limitation of this study was that it was conducted in a single orthopaedic unit in the United Kingdom. The generalisability of our findings is limited by geographic and cultural variation in surgical practice and patient footwear selection. We suggest that our findings can reasonably be extrapolated to other developed nations with similar patterns of footwear style and use.

We recommend an increased emphasis on pre-operative patient counselling; focused on the definition and clarification of the risks and benefits of surgery, particularly as they relate to footwear modification.

Female patients should be discouraged from re-introducing footwear that reproduces symptoms or delays their post-operative rehabilitation. However, those that express the desire to return to heeled footwear for personal or professional reasons should be advised that the majority of women are able to resume wearing these post-operatively. While this may discourage a small proportion of patients from undergoing surgery, it is likely to improve overall patient satisfaction in those that ultimately do undergo operation intervention.

## COMMENTS

### Background

Surgical intervention is indicated for symptomatic cases of hallux valgus unresponsive to conservative methods, with favourable reported outcomes. The return to various types of footwear post-operatively is reflective of the degree of correction achieved, and corresponds to patient satisfaction. Patients are expected to return to comfortable footwear post-operatively without significant residual symptoms. Many female patients will additionally attempt to return to high-heeled, narrow toe box shoes. However, minimal evidence exists to guide their expectations.

### Research frontiers

Few publications address post-operative footwear modification following hallux valgus surgery. However, the topic is one that frequently inquired about by patients during pre-operative counselling. Footwear outcomes have been reported to hold a strong correlation with global patient satisfaction and this warrants further investigation.

### Innovations and breakthroughs

The study focuses specifically on footwear modification following hallux valgus surgery, defining rates of return to comfortable and high-heeled narrow toe-box (HHNT) footwear and qualifying patterns of use. Additionally, the study is the first to stratify by age cohort and operation type.

### Applications

The authors' findings will help guide pre-operative patient counselling regarding footwear modification after hallux valgus surgery. This will enable clinicians to assist patients in setting realistic expectations over the end-points and timing of their functional recovery.

### Terminology

HHNT footwear was defined for the purposes of this study as: Tight-fitting shoes with a heel size  $\leq 3$  cm  $\pm$  a narrow toe-box.

### Peer-review

This is a well written study with straight forward methodology and results.

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## CD4-positive diffuse large B-cell lymphoma: A variant with aggressive clinical potential

Mohammad O Hussaini, Friederike H Kreisel, Anjum Hassan, TuDung T Nguyen, John L Frater

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### Abstract

CD4 expression is rare in diffuse large B-cell lymphoma (DLBCL), with 4 previously reported cases. Its significance is uncertain. We report five patients with CD4<sup>+</sup> DLBCL and one CD4<sup>+</sup> primary mediastinal large B-cell lymphoma. Cases were identified by searching the electronic database of the department; each was reviewed. Average age was 56 years. Neoplastic cells expressed CD20 (5/6 tested cases). BCL2/BCL6 expression were seen in 3/3 tested cases, suggesting a germinal center origin. Additionally, expression of T-cell antigens CD2 and CD5 was noted in 2/2 and CD7 in 1/1 tested case. CD3 was negative in all. Lymph nodes were commonly involved (67%). Patients received chemotherapy +/- radiation (6/6) and bone marrow transplant (2/6). Average survival was 44.2 mo. CD4 expression in DLBCL raises questions of lineage commitment. CD4<sup>+</sup> DLBCL is rare; care should be exercised not to diagnose these as T-cell lymphomas. A subset behaves aggressively.

**Key words:** CD4<sup>+</sup>; Diffuse large B-cell lymphoma; T-cell lymphoma; Lineage infidelity

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**Core tip:** Aberrant expression of T-cell antigens including CD4 in ALK-negative diffuse large B-cell lymphoma (DLBCL) is a rare phenomenon that raises interesting biological and diagnostic considerations. With regards to our series of DLBCLs expressing CD4, it appears that at least a subset may behave aggressively based on our data.

Hussaini MO, Kreisel FH, Hassan A, Nguyen TT, Frater JL. CD4-positive diffuse large B-cell lymphoma: A variant with aggressive clinical potential. *World J Methodol* 2016; 6(3): 181-186



## INTRODUCTION

Most cases of diffuse large B-cell lymphoma (DLBCL) show expression of B-cell antigens CD19, CD20, CD22, and CD79a. Variable expression of CD10, BCL-6, IRF4/MUM1, FOXP1, and less commonly CD5, is also observed. Surface/cytoplasmic light chain expression is usually present<sup>[1]</sup>.

The expression of T-cell associated antigens is not seen in benign lymphoid proliferations and is uncommon in B-cell non-Hodgkin lymphoma (B-NHL), most frequently occurring in the setting of chronic lymphocytic leukemia/small lymphocytic lymphoma<sup>[2]</sup>. In a series of 101 B-NHL, CD2, CD5, and CD7 expression was seen in only one quarter of cases<sup>[3]</sup>. CD8 (2%-3% of cases) expression has also been documented<sup>[4]</sup>. CD3 and CD4 expression, however, are exceedingly rare in DLBCL with only 4 cases of the latter reported in the world literature to the best of our knowledge<sup>[5-7]</sup>. Herein, we report a series of six cases of CD4<sup>+</sup> large B-cell lymphoma (LBCL) identified from our institution and review the pertinent literature to determine the incidence and possible mechanisms of aberrant T-cell antigen expression in B-NHL.

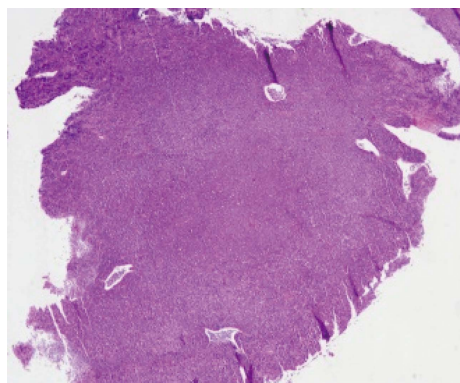
## LITERATURE

Permission for the study was granted by the Washington University (WU) Human Studies Committee. Consecutive cases diagnosed within a 21-year period in patients 0-99 years old were identified by searching the Copath database of the Division of Anatomic Pathology at Barnes Jewish Hospital/WU (WU/BJC) using the terms "large B-cell lymphoma" and "CD4" in any field. Each hit was then individually reviewed to identify cases of DLBCL with concomitant CD4 expression or aberrant expression of other T-cell antigens by immunohistochemistry or flow cytometry.

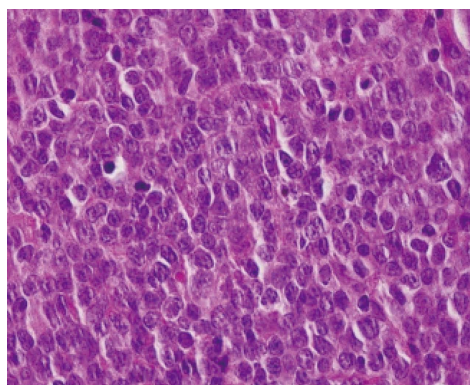
Clinical data were acquired from WU/BJC Clinical Desktop, CoPath, and Touchwork databases, and by clinician interview. Mortality data were also procured from the Social Security Death Index.

## RESULTS

Histologic and clinical features for all six cases are detailed in Table 1. Patients ranged in age from 22-79 years (average 54 years), and included 3 men and 3 women. Due to the retrospective nature of the study information about extent of disease at presentation was limited to three patients, and ranged from relatively limited disease in patients 1 and 2 to more extensive involvement in patient 5. Lymph node biopsies were obtained from the cervical or supraclavicular regions in 4 patients. In patient 1 tissue from the left maxillary



**Figure 1** Diffuse effacement of underlying tissue architecture by neoplastic cells in left maxillary sinus biopsy, patient 1 (hematoxylin and eosin, original magnification × 400).



**Figure 2** Large malignant cells with increased nuclear:cytoplasmic ratio, multiple nucleoli, irregular and nuclear contours arranged in sheets, case 1 (hematoxylin and eosin, original magnification × 1000).

sinus was biopsied, and in patient 6 the skin of the right leg was sampled.

Grossly, the tumors were noted to have a soft, grey/gelatinous appearance in two cases, and in one of these cases it was mistaken as a myxoma initially. In terms of immunophenotype, the neoplastic cells usually expressed CD45 (4/4 tested cases), CD20 (5/6 tested cases), CD19, and PAX5. BCL2, BCL6, and CD10 expression were also seen implying a germinal center origin (Figures 1-3). BCL2 and BCL6 expression were seen in 3/3 tested cases. CD4 was expressed in the vast majority of cells in 5/6 cases, and was positive in approximately 1/2 of the large cells in the biopsy from patient 4 (Figure 3). All cases were tested for CD3 expression by immunohistochemistry and were negative. With the exception of patient 4, the malignant cells in all patients were positive for CD20 immunohistochemistry. A monoclonal rearrangement of IgH by polymerase chain reaction (PCR) established the malignant lineage of the cells from patient 4. In addition to CD4, aberrant expression of T-cell antigens CD2 and CD5 was noted in two cases each and CD7 in one case. No TCR rearrangements were found in one tested case.

Five patients were diagnosed with DLBCL, including

**Table 1** Summary of histologic and clinical features of ALK-negative, CD4-positive diffuse large B-cell lymphomas

Case no.	Age/ sex	Diagnosis	Immunophenotype	Molecular findings	Gross description	Treatment	Clinical outcome	Site	Stage
1	73/M	DLBCL	CD20, BCL-2, CD10, CD5, CD43, BCL-6, and CD4		Soft, gelatinous appearing mass	R-CHOP × 4	Complete remission at 4 mo follow-up; free of disease at 6 mo follow-up	Left maxillary sinus	I-E
2	22/F	PMLBCL	CD45, CD20, CD79a, with variable expression of CD2, CD4, CD30, CD23, BCL-6 and BCL-2			12 cycles of R-VACOP-B; 2 cycles of R-ESHAP mediastinal radiation (40-50 Gy); matched, unrelated donor stem cell transplant with Bu/Cy conditioning; salvage chemotherapy with GND; SGN-35	Dead; 14 mo survival from disease discovery; Progressive disease	Supraclavicular lymph node	IIE-X-B
3	79/F	DLBCL and FL(3a)	CD45 (focal), CD20, CD3, CD21 (focal), Bcl-2, Pax-5, subset expression of CD2, CD4, CD5, CD7, CD8 Bcl-6, MUM1, and CD10			R-CHOP	Dead; 6 mo survival from disease diagnosis	Left neck lymph node	
4	67/M	CLL/SLL with transformation to LBCL	CD45, CD30, EMA, CD4 (subset), and CD43, rare weak CD2	46, XY <sup>[18]</sup> ; clonal IGH rearranged; IGVH unmutated; TCR gamma rearrangement negative		Fludarabine and cytoxan × 3; fludarabine, Rituxan, and mitoxantrone × 2; R-CHOP × 4; R-CHOP; BEAM and auto transplant	Dead; 15 mo survival; Progressive disease	Left subclavicular lymph node	
5	26/M	B-cell large cell lymphoma	CD20, CD30 (weak), CD4 (subset)			R-CHOP × 5, field radiotherapy	No evidence of relapse at 72 mo; lost to follow up	Left posterior cervical lymph node	IV-A-E
6	55/F	Malignant lymphoma, diffuse cleaved large cell type, with B-cell differentiation	CD20, MB-2, CD4, BCL2, and CD43 PCNA				Lost to follow-up	Right leg skin	

CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma; CD: Cluster of differentiation; DLBCL: Diffuse large B-cell lymphoma; F: Female; M: Male; PMLBCL: Primary mediastinal large B-cell lymphoma; CHOP: Rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate; R-ESHAP: Rituximab, etoposide, methylprednisolone, high-dose cytarabine, and cisplatin; VACOP: Vinblastine, adriamycin, cyclophosphamide, etoposide, prednisone, bleomycin; GND: Gemcitabine, vinorelbine, and doxorubicin; SGN-35: Brentuximab vedotin; BEAM: Carmustine, etoposide, cytarabine, and melphalan; FL: Follicular lymphoma.

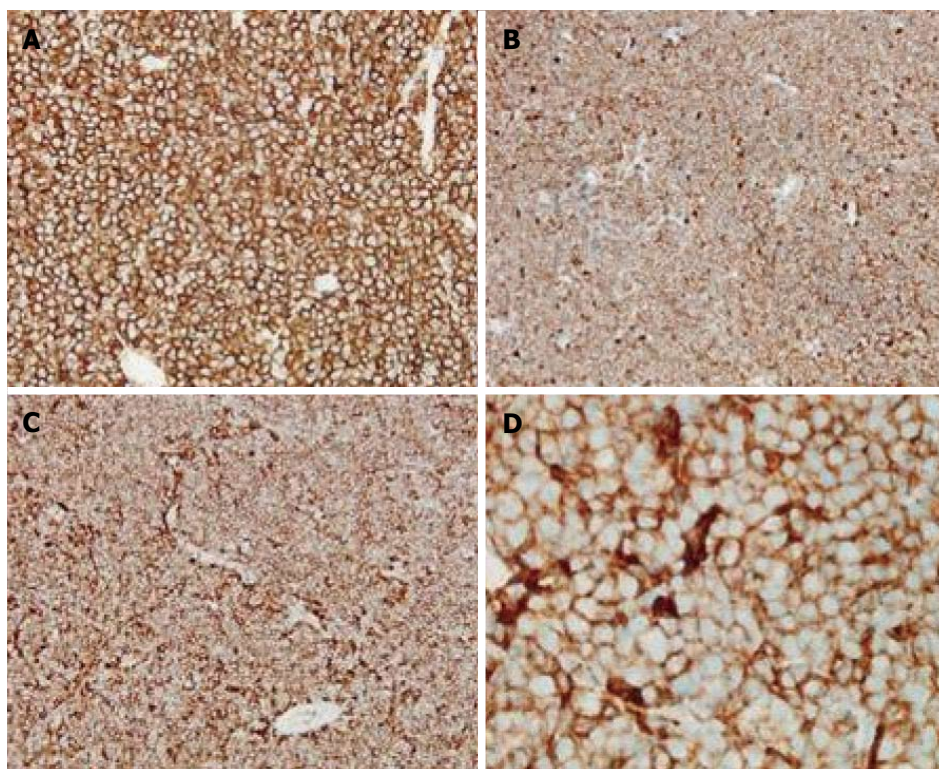
one (patient 4) in which the DLBCL represented a Richter transformation of a prior chronic lymphocytic leukemia/small lymphocytic lymphoma and one (patient 3) with DLBCL and associated grade 3a follicular lymphoma. Patient 4 presented with primary mediastinal large B-cell lymphoma.

Clinical follow-up was available for 5/6 patients, who were all treated with multiagent chemotherapy. Two patients received stem cell transplants. Two patients had an initial response until lost to further follow-up at

6 and 72 mo, respectively, and 3 died of progressive disease between 6-15 mo after original diagnosis. Average survival was 44.2 mo overall, and 11.7 mo for those who died.

## CONCLUSION

Herein, we report the largest series of ALK-negative CD4-positive DLBCL. Five cases were DLBCL, NOS and one was an example of PMLBCL. In our cohort (*n*



**Figure 3** Immunohistochemistry results (clockwise). A: CD20 (original magnification  $\times 200$ ); B: CD5 (original magnification  $\times 200$ ); C: CD4 (original magnification  $\times 200$ ); D: CD4 (original magnification  $\times 1000$ ).

= 6), the average patient age was 56 years (range: 22-79). There was no sex predilection (3 males, 3 females). Lymph nodes were the most common site of involvement (67%). All patients received multi-agent chemotherapy, and two patients underwent stem cell transplantation. Average overall survival ( $n = 6$ ) was 44.2 mo. For those who died ( $n = 3$ ), average survival was only 11.7 mo.

The aberrant expression of T-cell associated antigens is not seen in benign lymphoid proliferations and is rather uncommon in B-NHL<sup>[7,8]</sup>. Nonetheless, aberrant expression of CD5, CD2, CD3, CD4, CD7, CD8, and CD45RO (UCHL-1) have all been reported in B-cell lymphomas. Most commonly, aberrant expression of T-cell antigens is seen in CLL and mantle cell lymphoma. Overall, the reported frequency of T-cell antigen expression in B-NHL ranges from 19% to 25%<sup>[8]</sup>. CD4 expression is unusual in B-NHL. It has been reported in plasmablastic lymphoma (3 cases), DLBCL associated with chronic inflammation/pyothorax-associated lymphoma (1 case), and CLL (1 case)<sup>[9]</sup>. Interestingly, CD4 positivity is frequently seen in ALK<sup>+</sup> DLBCL with one series citing a frequency of 64% (14/22 cases) in these rather uncommon neoplasms<sup>[10]</sup>. In DLBCL, NOS, CD4 expression is particularly rare with only 4 cases reported in the world literature to the best of our knowledge<sup>[6,8,11]</sup>.

Review of the literature shows four prior reports or ALK-negative, CD4-positive DLBCL (Table 2). The earliest case was identified by Olack *et al.*<sup>[8]</sup> who described a case of DLBCL that was positive for CD19,

surface kappa light chain, CD4, and CD7 by flow cytometry. The patient was an 81-year-old man with moderate lymphadenopathy but no organomegaly or bone marrow involvement. He had stage III disease and was lost to follow up after 13 mo of stable disease.

The next patient was an 82-year-old man who was found to have left cervical and left inguinal lymph node swelling. Biopsy of an involved lymph node showed DLBCL. Dual staining confirmed that the cells of interest were positive for both CD4 and CD19. Complete remission was achieved after five cycles of rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate (R-CHOP)<sup>[11]</sup>.

2010, Arrondini *et al.*<sup>[6]</sup> reported 2 cases of CD4<sup>+</sup> DLBCL occurring in the small bowel. The first of these was a 55-year-old woman who presented with lymphoma entrapping the last part of the ileum, pancreas, and omentum. Molecular studies showed a clonal IgH rearrangement and no BCL2 rearrangement. The patient received dose-escalated (Mega) CHOP but two weeks later required a laparotomy for resection of 60 cm of ulcerated and perforated small bowel. The patient received 5 more cycles of R-MegaCHOP and achieved complete remission.

The second case reported by Arrondini *et al.*<sup>[6]</sup> involved a 73-year-old man who presented with DLBCL involving the ileum. A staging bone marrow biopsy was negative. The patient was treated with adjuvant chemotherapy. Two years later, he presented with an enlargement of the pancreatic head and a mass involving the right



**Table 2** Summary of ALK-negative, CD4-positive diffuse large B-cell lymphomas reported in the literature

Case no.	Age/sex	Diagnosis	Immunophenotype	Molecular findings	Gross description	Treatment	Clinical outcome	Site	Stage	Ref.
1	81/F	DLBCL	CD19, CD4, CD7				Lost to follow-up after 13 mo of stable disease	N/A	III	[2]
2	82/M	DLBCL	CD4+, CD5+, CD19+, CD20+, CD23+, CD25+, kappa+			R-CHOP × 5	Complete remission after chemotherapy	Left cervical and left inguinal lymph node		[11]
3	55/F	DLBCL	CD20 (weak), BCL2, PAX5, surface kappa, MUM1, and CD4	Clonal <i>IgH</i> gene rearrangement and no <i>BCL2</i> gene rearrangement	Uniformly soft, greyish tissue	Mega Chop; R-Mega CHOP × 5	Complete remission after chemotherapy	Ileum		[6]
4	73/M	DLBCL	CD45 (dim), CD19, PAX5, CD20, CD10, BCL6, BCL2, surface lambda light chain, and CD4	Clonal <i>IgH</i> gene rearrangement and a <i>BCL2</i> gene rearrangement		Adjuvant chemotherapy		Ileum		[6]

CD: Cluster of differentiation; DLBCL: Diffuse large B-cell lymphoma; F: Female; M: Male; N/A: Not available; CHOP: Rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, mega.

adrenal gland and superior pole of the right kidney. Retroperitoneal lymph-node fine needle aspiration showed numerous large lymphoid cells with centrally-located nucleoli and vesicular chromatin.

Aberrant expression of CD4 in DLBCL is a rare but intriguing finding that raises questions of lineage fidelity and the biology underlying such aberrant expression. Under physiological conditions, lineage commitment and differentiation are considered unidirectional and irreversible processes<sup>[12]</sup>. That is to say, a lymphocyte committed to the B-cell program is destined to become a B-cell, one that we do not normally expect to dedifferentiate to an immature form. So how then does one explain aberrant T-cell antigen expression in B-cell lymphomas? Various hypotheses, some of which challenge this paradigm, have been advanced to account for this unusual phenomenon.

Some believe that aberrant expression is the result of neoplastic transformation at stem cell level before commitment to either B or T-cell differentiation<sup>[13,14]</sup>. Others postulate expansion of a normal subpopulation expressing T cell antigens [CD5<sup>+</sup> B1 cells, CD2(+) B cells, CD7(+) B cells]<sup>[8,15]</sup>. It is also possible that deregulated control of gene expression in malignant B cells leads to the activation of some otherwise silent or repressed genes of T-cell differentiation<sup>[8,13,15-18]</sup>. For example, deregulated/damaged PAX5 might downregulate B-cell antigens and lead to aberrant expression of a T-cell antigen such as CD4. This notion is corroborated by murine models in which conditional deletion of PAX5 resulted in dedifferentiation of mature peripheral B cells into early uncommitted progenitors that were able to rescue T lymphopoiesis in the thymus of T-cell-deficient mice<sup>[12]</sup>.

The import of T-cell antigen expression in B-NHL lies beyond its identification as a biological curiosity or its role in helping us understand lymphopoiesis, and in turn

lymphomagenesis. There are practical implications as well. Particularly, recognition of T-cell antigen expression in B-NHL is important to avoid erroneous diagnostic consideration of a T-cell malignancy, a potential pitfall that is potentiated by sometimes weak expression of B-cell defining markers. Given that T-cell markers are not routinely performed in cases of DLBCL, the actual incidence of CD4 positivity may actually be higher than cited in the literature. However, results from sizeable series looking at T-cell antigen expression in B-NHL would argue against this possibility and suggest that CD4 expression is in fact rare<sup>[3]</sup>.

We must also consider whether there are any prognostic implications. The data are not entirely clear in this regard. There are isolated reports showing worse outcomes for T-Ag (+) B-NHLs. Some report increased extranodal involvement and a higher International Prognostic Index (high and high intermediate) score in such cases<sup>[3]</sup>. On the other hand, Olack *et al*<sup>[8]</sup> did not find any difference when T-Ag (+) B-NHLs were compared to their normal B-NHL counterparts. With regards to our series of DLBCLs expressing CD4, it appears that at least a subset may behave aggressively based on our data. In summary, aberrant CD4 expression in ALK-negative DLBCL is a rare, but documented, phenomenon that raises interesting biological and diagnostic considerations.

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## Modified skin bridge technique for ilio-inguinal lymph node dissection: A forgotten technique revisited

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### Abstract

Ilio-inguinal lymph node dissection (IILD) is a commonly performed surgical procedure for a number of malignant conditions involving mainly the male and female genitalia, and the skin; however the postoperative morbidity of IILD, due to high frequency of flap necrosis, wound infection and seroma formation, has always been a major concern for the surgeons. The aim of the study is to highlight a modified skin bridge technique of IILD using two parallel curvilinear incisions to minimize postoperative skin flap necrosis. This technique was successfully employed in 38 IILD during May 2012 to November 2013. None of the patient had flap necrosis. Two patients developed seroma while another two patients had superficial surgical site infection; they were managed conservatively. Modified skin bridge technique for IILD is an effective method to minimize flap necrosis without compromising the oncological safety.

**Key words:** Skin bridge technique; Wound infection; Ilio-inguinal lymphadenectomy; Flap necrosis

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**Core tip:** Ilio-inguinal lymph node dissection (IILD) has classically been associated with high postoperative complications which include flap necrosis, wound infection, seroma formation, and lymphedema. A modified skin bridge technique of performing IILD aims to minimize the postoperative wound complications. It is an effective method to minimize flap necrosis without compromising adequate oncologically safe dissection.

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bridge technique for ilio-inguinal lymph node dissection: A forgotten technique revisited. *World J Methodol* 2016; 6(3): 187-189 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i3/187.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i3.187>

## INTRODUCTION

Ilio-inguinal lymph node dissection (IILD) has classically been associated with high postoperative complications which include flap necrosis, wound infection, seroma formation, and lymphedema. Although a number of modifications of surgical techniques to perform IILD have been reported in the literature, frequency of various complications remains high. In a recent review of IILD, Hegarty *et al*<sup>[1]</sup> reported that frequency of skin flap necrosis varies from 7.5% to 61% in radical IILD; other major complications of IILD include infection (7.5% to 14.2%), seroma formation (5% to 13.8%) and lymphedema (2.5% to 5.2%). In 1972, Fraley *et al*<sup>[2]</sup> described a new technique "skin bridge technique" for IILD to reduce postoperative wound complications; however, this technique failed to gain popularity among the surgeons. We, herein, present our modified skin bridge technique which aims to lessen the frequency of postoperative skin flap necrosis.

## OPERATIVE TECHNIQUE OF MODIFIED SKIN BRIDGE TECHNIQUE

The patient was positioned supine with abduction and external rotation at hip joints (Figure 1). This technique entailed two curvilinear incisions: An inguinal incision, of approximately 7 cm length, 4 cm below and parallel to the inguinal ligament; and an iliac incision, of 5-7 cm length, 4 cm above and parallel to the inguinal ligament (Figure 2A). During the inguinal part of dissection, the skin flap was raised preserving the subcutaneous fat, just superficial to Scarpa's fascia, with flap (Figure 2B); the lateral limit of dissection was the medial border of Sartorius and the medial limit was the lateral border of adductor longus, the upper limit was 2-3 cm above the inguinal ligament, and the lower limit was the apex of the Scarpa's triangle. During the iliac part of the dissection - the external oblique muscle was divided along the skin incision, and the internal oblique and transverses abdominis muscles were split along the muscle fibres. The retroperitoneal space was entered thereafter. Inguinal and iliac lymphadenectomy is completed as per the standard technique. The margins of the inguinal incisions were routinely freshened before closure. Sartorius flap was routinely used to cover the femoral vessels in all patients. One closed suction drain was placed in the inguinal region; no drain was used for iliac dissection area (Figure 3). A light pressure dressing was done. All the patients were prescribed elastic stockings within eight hours of the surgery.



Figure 1 Position of the patient, supine with abduction and external rotation at both hip joints.

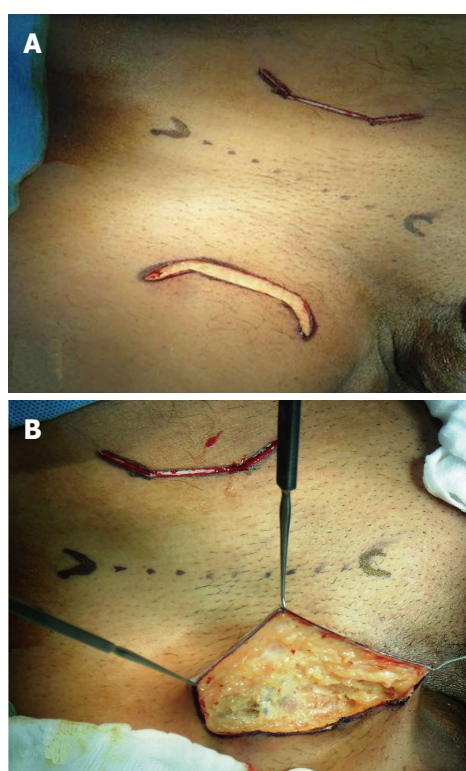


Figure 2 Two parallel curvilinear incisions technique: One inguinal incision, another iliac incision (A), and during inguinal part of dissection, the skin flap is raised preserving the subcutaneous fat, superficial to scarpa's fascia (B).

Antithrombotic prophylaxis was not routinely prescribed. The patients were discharged on 2<sup>nd</sup> postoperative days after having been explained and taught surgical site care and how to measure daily drain output. The drain was removed in an out-patient clinic after the drain output decreased to less than 40 mL/d. All patients receive perioperative antibiotics for five days or till drainage tube was not removed.

## RESEARCH RESULT

This technique was successfully employed in 38 IILD



Figure 3 Immediate postoperative photograph.

during May 2012 to November 2013. There was no perioperative mortality. None of the patient had flap necrosis. Six patients developed seroma while another two had superficial surgical site infection; they were managed conservatively. For a unilateral ilio-inguinal lymphadenectomy, the median lymph nodes harvested were 10 (interquartile range 7-15) while the median operative time and blood loss was 50 min (interquartile range 45-60) and 15 mL (interquartile range 10-25) respectively.

## DISCUSSION

IILD has been traditionally an integral part of management of male and female genital cancers, and skin cancers; however, it is associated with considerable post operative morbidity and high frequency of ischemic necrosis of skin flaps. These complications have reduced over a period of time with certain technical modifications as it is evident from two publications from a single institute in south India<sup>[3,4]</sup>. In a large series of 174 IILDs, performed from 1962 to 1990, Ravi<sup>[3]</sup> reported that postoperative complications of IILD were partial skin flap necrosis in 64%, wound infection in 14%, seroma formation in 9%, and limb lymphedema in 29% of dissections. Another paper from the same institute<sup>[4]</sup>, published 14 years later highlighted a lower rate of flap necrosis (19.8%) in 202 IILDs performed from 1987 to 1998. Though there was a reduction in the rate of flap necrosis in IILDs, it continued to be considerably high (19.8%). The high postoperative morbidity associated with IILD is at the helm of many controversies: Whether the extent of lymphadenectomy can be curtailed in early cancer? Whether should one go for sentinel node biopsy? Whether there is any role for prophylactic lymphadenectomy at all? And what is the role of whole

gamut of new minimally invasive techniques of IILD. We tried to address to problem of skin flap necrosis with our innovative modified skin bridge technique using two parallel curvilinear incisions.

The blood supply of the skin flap is derived from the three superficial branches of the femoral artery namely - superficial external pudendal, superficial epigastric and superficial circumflex iliac. These vessels are usually divided during conventional technique of groin dissection, and so the vascularity of the skin flaps rely solely upon the anastomotic branches of these vessels forming a microvascular arcade which lies in the camper's fascia and runs parallel to inguinal ligament<sup>[5]</sup>. This anatomical fact is the basis of our technique as both incisions remain parallel to the inguinal ligament, and thus spare the above mentioned vessels which run superficial to Scarpa's fascia. The curves at the ends of the curvilinear incisions define the medial and lateral boundary of the dissection; an overzealous dissection beyond the limits has been a crucial factor which can jeopardize the vascularity of skin flaps and result in its necrosis<sup>[3]</sup>.

There are some minor modifications that we made in our technique compared to what was described originally by Fraley and Hutchens<sup>[2]</sup>: (1) relatively smaller skin incisions; (2) routine transposition of Sartorius muscle to cover the femoral vessels; (3) no routine femoral hernia repair; (4) no *en bloc* dissection of iliac and inguinal lymph nodes; and (5) routine freshening of both skin edges of inguinal incision.

## CONCLUSION

Modified skin bridge technique for IILD is an effective method to minimize flap necrosis without compromising oncological safety.

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Basic Study

## Adjuvant role of *Pseudomonas* flagellin for *Acinetobacter baumannii* biofilm associated protein

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**Author contributions:** Rasooli I contributed to the conception and design of the study, or acquisition of data, or analysis and interpretation of data; Sefidi MD carried out the experimental part of the project; Owlia P revised it critically for important intellectual content; Talei D carried out the statistical analyses; Astaneh SDA supervised the experiments; Nazarian S drafted the article; and final approval of the version to be submitted was made by Rasooli I.

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**Institutional animal care and use committee statement:** The animal care protocol was approved by Shahed University. 4-6 wk old (16-22 g) BALB/c mice procured from the Razi Institute, Tehran, Iran were housed in clean standard animal care facility of Shahed University. The research was carried out in compliance with the Animal Welfare Act and regulations related to experiments involving animals. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

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**Data sharing statement:** Technical details and dataset available from the corresponding author at [rasooli@shahed.ac.ir](mailto:rasooli@shahed.ac.ir). No additional data are available.

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## Abstract

### AIM

To study immunogenicity of *Pseudomonas* N terminal flagellin as an adjuvant for *Acinetobacter baumannii* (A. *baumannii*) biofilm associated protein (Bap).

### METHODS

The N terminal flagellin gene was amplified. The pET28a (+) and polymerase chain reaction products were

digested with HindIII and EcoR I. The ligation of N terminal flagellin into pET28a (+) was performed using T4 DNA ligase and was then transformed into *Escherichia coli* BL21 (DE3) as a suitable expression host. pET28a (+) vector harboring a conserved region of Bap from our previous work was used. The recombinant proteins were expressed, analyzed by SDS-PAGE method and was purified by affinity chromatography with His-Tag residues followed by confirmation with western blotting. Mice were immunized with recombinant N terminal flagellin and Bap subunits. The immunized animals were intranasally (i.n) challenged with *A. baumannii* and *Pseudomonas aeruginosa* (*P. aeruginosa*).

## RESULTS

The flagellin enhanced the immunogenicity of Bap causing an increase in specific IgG titers in serum ( $P < 0.001$ ). Internal organs, *i.e.*, liver, lung and spleen of the Bap-Flagellin immunized group challenged with *A. baumannii* showed significantly lower bacterial load compared to the control group. The bacterial loads were studied in internal organs. *A. baumannii* infected immunized animals with Bap-Flagellin exhibited internal organs with minor bacterial load while *P. aeruginosa* PAO1 infected group showed heavy bacterial load of  $(4.3 \pm 0.12) \times 10^6$ ,  $(1.1 \pm 0.01) \times 10^6$  and  $(2.2 \pm 0.22) \times 10^6$  per gram of lungs, liver and spleen respectively. Bacterial loads were detected per gram of lungs, liver and spleen of the mice group immunized with Bap were  $(1.2 \pm 0.06) \times 10^7$ ,  $(11.1 \pm 0.041) \times 10^5$  and  $(3.6 \pm 0.42) \times 10^6$  respectively. *In vivo* neutralization assay indicated that all experimental mice groups, except for Flagellin administered group was significantly ( $P < 0.05$ ) protected against *A. baumannii*.

## CONCLUSION

These results demonstrate that *P. aeruginosa* Flagellin as an adjuvant for Bap *A. baumannii* could be a useful model to evaluate new vaccine against *A. baumannii*.

**Key words:** *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; Vaccine; Immunogen; Biofilm associated protein

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**Core tip:** The increasing frequency of *Acinetobacter baumannii* (*A. baumannii*) infections and its drug resistance challenge health authorities. Flagellin is an effective immune activator stimulating various biologic functions identified by Toll like receptor 5. Conserved regions of biofilm associated protein (Bap) have already been identified and their immunoprotectivity against *A. baumannii* have been established. In order to enhance their immunogenic activities, we designed a study on adjuvant role of flagellin from *Pseudomonas aeruginosa* for Bap.

S. Adjuvant role of *Pseudomonas* flagellin for *Acinetobacter baumannii* biofilm associated protein. *World J Methodol* 2016; 6(3): 190-199 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i3/190.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i3.190>

## INTRODUCTION

*Acinetobacter baumannii* (*A. baumannii*) an opportunistic pathogen, causes severe infections of major concerns<sup>[1,2]</sup>. It survives on harsh dreadful environment such as medical devices. This ability has been related to forming multicellular complex named biofilm on abiotic and biotic surfaces<sup>[3,4]</sup>. The *A. baumannii* survival has also been contributed with clinical strains resistance to antimicrobial stressors and desiccation<sup>[5,6]</sup>. Formation by *A. baumannii* clinical strains of biofilms on abiotic surfaces has been documented<sup>[5]</sup>. Production of factors like poly-β-1,6-*N*-acetyl glucosamine (PNAG), in different bacteria that produce PNAG could also act as a major virulence factor for emerging biofilm-dependent pathogens<sup>[7]</sup>. Biofilms are encapsulated bacterial constructions within exopolysaccharide, a polymeric matrix important in medicine<sup>[8]</sup>. Identifying a biofilm associated protein (Bap) homologue of staphylococcus<sup>[9]</sup>, in an isolate of *A. baumannii* from bloodstream showed that Bap is the most important factor in *A. baumannii* biofilm formation<sup>[10]</sup>. The role of high molecular weight of superficial Bap conferring biofilm formation capacity upon bacterial attachment and infection has been reported<sup>[10]</sup>. Bap *A. baumannii* is one of the biggest and the most acidic bacterial proteins with a predicted pI-3 is composed of about 8620 amino acids(aa). Seven tandem repeats are the major functional and conserved regions components of Bap<sup>[11]</sup>. Using adjuvants and immune modulators during vaccination helped to increase immune responses<sup>[12]</sup>. Adjuvant can enhance host response against an immunogen or a vaccine without imparting antigenic effect. Various substances acting as vaccine delivery vehicles such as mineral salts, particulate and surfactants or liposomes and virosomes have been extensively used as adjuvants<sup>[13]</sup>. Pattern recognition receptors called "Toll like receptors (TLRs)" are present on various types of microbial cells<sup>[14]</sup>. TLR5 recognizes flagellin, a potent immune activator stimulating diverse biological functions. In a classic study, a recombinant fusion protein strategy was used with TLR5s like Flagellin to show TLR5 role in the development of vaccine<sup>[15]</sup>. N-terminal region amino acids of *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 flagellin was administrated to play significant binding role to TLR5. Specific involvement of different amino acids in TLR5-flagellin interactions was particularly predicted<sup>[16]</sup>, so researches show significant role of N-flagellin terminal region in immunization and can play as well as whole flagellin. The present study was designed to examine the role of *Pseudomonas* flagellin as an adjuvant to Bap

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against infections caused by *A. baumannii*.

## MATERIALS AND METHODS

### Materials

The kits for extraction of plasmids for purification of gels were purchased from a local dealer. Synthesis of the designed primers was done by Gene Fanavaran (Tehran, Iran). Standard quality restriction enzymes, Nickel Nitritotriacetic Acid (Ni-NTA), T4 DNA ligase, nitrocellulose membrane, anti-polyhistidine antibodies, anti-mouse HRP conjugated IgG, microtiter plates, and other standard chemicals and reagents were procured from local market.

### Bacteria

*A. baumannii* (ATCC 19606), *P. aeruginosa* (PAO1) and *Escherichia coli* (*E. coli*) BL21 (DE3) grown in Luria Bertani (LB) medium on a shaker at 37 °C /220 rpm were used.

### Gene amplification and plasmid construction

The N terminal Flagellin gene (Gen Bank accession No.: AGY69124.1) with 1-180aa of the mature Flagellin protein weight of 53.79 kDa from *P. aeruginosa* was amplified by polymerase chain reaction (PCR) using its genomic DNA. N terminal F (5'-ATATGAATTCATGGCCCTTACAGT-3') and N terminal R (5'-TATAAAGCTTTTAACCGCTGATCT-3') primers. The F primer contained EcoRI site and R primers had HindIII site. PCR conditions started at 95 °C/ 5 min followed by 35 cycles at 94 °C/30 s, 58 °C/1 min, 72 °C /90 s, and a 72 °C for 5 min as the final extension. One percent agarose gel was used to analyze the amplified fragments. Double Digestion of PET28a (+) vector and PCR products was carried out and T4 DNA ligase was used for ligation of N terminal Flagellin into pET28a (+). Transformation of the recombinant DNA plasmids, N Flagellin, pET28a into *E. coli* BL21 was then carried out.

### Protein expression and purification

Vector harbouring pET28a-n Flagellin construct was incubated for 3 h at 37 °C in LB agar incorporated with 70 µg/mL kanamycin to an OD<sub>620</sub> of 0.6. Induction was brought about with 1 mmol IPTG for 3 h at 37 °C. The cell pellet was collected after 10 min centrifugation at 5000 rpm and re-suspended in denaturation lysis/ binding buffer (buffer B). Lysate sonication at 200 w with a 10 s cooling time between each burst was done for 6 times. The lysate was then centrifuged at 14000 rpm at 4 °C for 20 min to pellet the cell debris. The affinity Ni-NTA agarose column loaded with supernatant was washed with denaturing buffer (buffer C, pH = 6.3), and denaturing elution buffer D (pH = 5.9). The recombinant Bap subunit was then eluted using denaturing elution buffer E (pH = 4.5). Eight mol/L urea was present in all buffer solutions. Protein analysis was carried out by 10% SDS-PAGE. Dialysis was performed in descending order against PBS (pH = 7.4) containing 6 to 0 mol/L urea. Huleatt *et al.*<sup>[15]</sup>

method was employed to determine the purified protein concentration. Bovine serum albumin (BSA) was used as a standard protein. Production of the recombinant Bap and its purification was according to Fattahian *et al.*<sup>[17]</sup>.

### Western blotting

Western blotting was performed to confirm the expression of N-flagellin and Bap. Anti-His. 0.5 mg from each of purified recombinant Bap subunit was adsorbed on a nitrocellulose strip and dried. The transformed lysates from uninduced cell were used as control. The nitrocellulose strip was incubated for 1 h at room temperature with gentle shaking in the blocking buffer containing 5% skim milk. The strip was then subjected to three washes with phosphate buffered saline containing Tween 20 (PBST). Diluted anti-His conjugated was incubated with 1:8000 dilution of horseradish peroxidase for one hour. The strip was then subjected to three washes of 5 min each with PBST. Membrane visualization with the substrate diaminobenzidine was complete by observing brownish dots. Color development was terminated by washing with PBST.

### Animal immunization

Sixty BALB/c mice were divided equally in six groups: (1) the first group was administered with four 20 µg doses of the recombinant Bap; (2) the second group was administered with four 20 µg doses of the recombinant N Flagellin; (3) a single 20 µg dose of recombinant Bap followed by administration of N-flagellin in subsequent vaccinations was administration plan for the third group; (4) the fourth group received a single dose of 20 µg of recombinant N-flagellin followed by administration of Bap in subsequent vaccinations; (5) the fifth group received combination of Bap and N-flagellin simultaneously in all the vaccinations; and (6) the control group received combination of PBS and Freund's adjuvant.

All groups except group (5), first received complete Freund's adjuvant only. The subsequent vaccinations were with the incomplete Freund's adjuvant emulsified recombinant proteins. Ten days after the second dose the blood samples were collected through infra-orbital plexus.

### ELISA with recombinant Bap and N-flagellin subunit

The coating solution was used to dilute the recombinant Bap and N-flagellin subunit to an optimal concentration of 20 µg/mL to coat a 96-well plate. Each well was added with 100 µL of the resulting solution followed by incubation at 4 °C for 12-18 h. The wells were washed once with 0.05% Tween 20 (PBST) incorporated PBS to block the unoccupied sites. The plate was then incubated for 1 h at 37 °C with 100 µL of PBST + 5% skimmed milk. The plates were washed 3 times with PBST. Serial dilutions of 1:100 to 1:1600 from each serum were added to the wells. The plates were incubated at 37 °C for 1 h followed by 3 washes as described above. One hundred microlitre of 1:1000 horseradish peroxidase

- conjugated secondary antibody diluted in PBST was added to each well and the plates were incubated at 37 °C for one hour followed by three washes with PBST and incubation with 100 µL per well of TMB (3,3',5,5'-tetramethylbenzidine solution) substrate until a desired absorbance was reached. Addition of 2 mol/L H<sub>2</sub>SO<sub>4</sub> stopped the reaction. Sample absorbance at 450 nm was read on an ELISA plate reader.

### Cell ELISA

The clinical *A. baumannii* isolates incubated overnight in LB broth were resuspended in PBS to an OD<sub>620</sub> of 0.3<sup>[17]</sup>. Fifty microlitres of the bacterial suspension was added to each well in 96-well plates and dried at 37 °C. The plates were washed five times with after blocking with 200 µL of PBS with 5% skimmed milk for 1 h. This was followed by incubation with different dilutions of immunized mice sera. The plates were then incubated with HRP conjugated secondary antibody for one hour. PBS was used to wash the plates for five times. One hundred microlitres of TMB substrate was added per well and incubated until desired absorbance. The reactions were stopped with 2 mol/L H<sub>2</sub>SO<sub>4</sub> and OD<sub>450</sub> was read.

### Fifty percent lethal dose (LD<sub>50</sub>) determination

Cyclophosphamide (Cy) regimen (150 mg/kg of body weight) was used to bring about neutropenia of short duration but able to facilitate the onset of the infectious process. LD<sub>50</sub> was determined with intranasal (i.n) administration of *A. baumannii* at 10<sup>9</sup> CFU concentration 4 d after treatment of five BALB/c mice with Cyclophosphamide. PBS was administered as a control instead of bacteria. General anesthesia was brought about by intraperitoneal (i.p) injection of 0.2 mL of 1.3 mg/mL xylazine and 6.7 mg/mL of ketamine in 0.9% saline. This was 100-200 mg Ketamine and 5-16 mg Xylazine per kilogram body weight.

### Neutralization assay

Antisera to the Bap and Bap-Flagellin proteins were raised by injecting 20 µg of Bap and Bap-Flagellin per animal in BALB/c mice. Lethal dose (10<sup>6</sup> CFU) of *A. baumannii* diluted to 1:400 with PBS was maintained at 37 °C for 30 min. Neutralization test was carried out by peritoneal injection of lethal dose of *A. baumannii* to the mice groups of five animals per group. In order to rule out interfering role of natural antibodies in mice sera in conferring any resistance to mice against *A. baumannii*, a lethal dose of *A. baumannii* and PBS was administered to mice groups as control<sup>[18]</sup>. Mortality rate was monitored for 48 h.

### Challenge of mice

Mice immunized with Bap and Bap-flagellin were divided into two groups. The groups were challenged with *A. baumannii* and *P. aeruginosa* PAO1. The mice were observed for mortality for two days. The animals were

sacrificed after 48 h unless they died earlier. The microbial challenge or passive immunization were performed in mice groups treated with Cy. For bacterial challenge, i.n. administration with 20 µL of *P. aeruginosa* PAO1 or 20 µL of *A. baumannii* (10 µL/nosril) was carried out<sup>[19]</sup>. Morbidity and mortality were watched over 48 h.

### Statistical analysis

The experimental data in triplicates were expressed as mean ± SD. In order to calculate *P* values and to determine the significance of differences in the experimental groups, Student's *t* test was used. *P* < 0.05 was considered as significant for the combined injection of Bap and Flagellin.

## RESULTS

### Bap and N terminal flagellin gene

N terminal flagellin gene of *P. aeruginosa* (PAO1) was successfully amplified. The amplified gene was revealed on agarose gel (1%). N-flagellin gene was cloned into pET28a (+). The N-flagellin and Bap were expressed in *E. coli* BL21 (DE3). The recombinant proteins were confirmed by SDS-PAGE. The presence of 42 kDa (Bap) and 7 kDa (Flagellin) proteins in the eluted fractions was revealed by SDS-PAGE analysis. Western Blotting was used to confirm the expression of recombinant proteins using anti-His-tag antibodies (Figure 1).

### Recombinant proteins' immunogenic properties

Animals were observed healthy with no post immunization signs of abnormalities. Significant (*P* < 0.05) levels of IgG were noted in immunized sera compared to control mice. Increased antibody titer was noted after the third booster dose. No specific antibody was detected in sera samples from mice that received adjuvant and PBS. No significant difference was observed in the sera from combined administration of both proteins as compared to that of the single Bap injections (Figure 2A and E). Significant (*P* < 0.001) increase of antibody titer took place after the third booster, whereas animals administered with adjuvant and PBS or just Flagellin serving as control exhibited no Bap-specific antibodies in their sera.

### Cell ELISA

The antibody raised against Bap subunit reacted with *A. baumannii*. OD values detected were significant compared to control (Figure 3).

### Animal challenge with *A. baumannii* and *P. aeruginosa*

2.5 × 10<sup>9</sup> CFU/mL was determined as LD<sub>50</sub> per mouse via intranasal administration. Rendering mice immunocompromised by prior treatment with Cy makes them susceptible to pneumonia. This susceptibility is accompanied by a drop in the LD<sub>50</sub> after a challenge with 1000 CFU of a *P. aeruginosa* PAO1 or *A. baumannii* causing lethality. The control group died within the first 24 h of challenge. The Bap immunized groups resisted *A.*



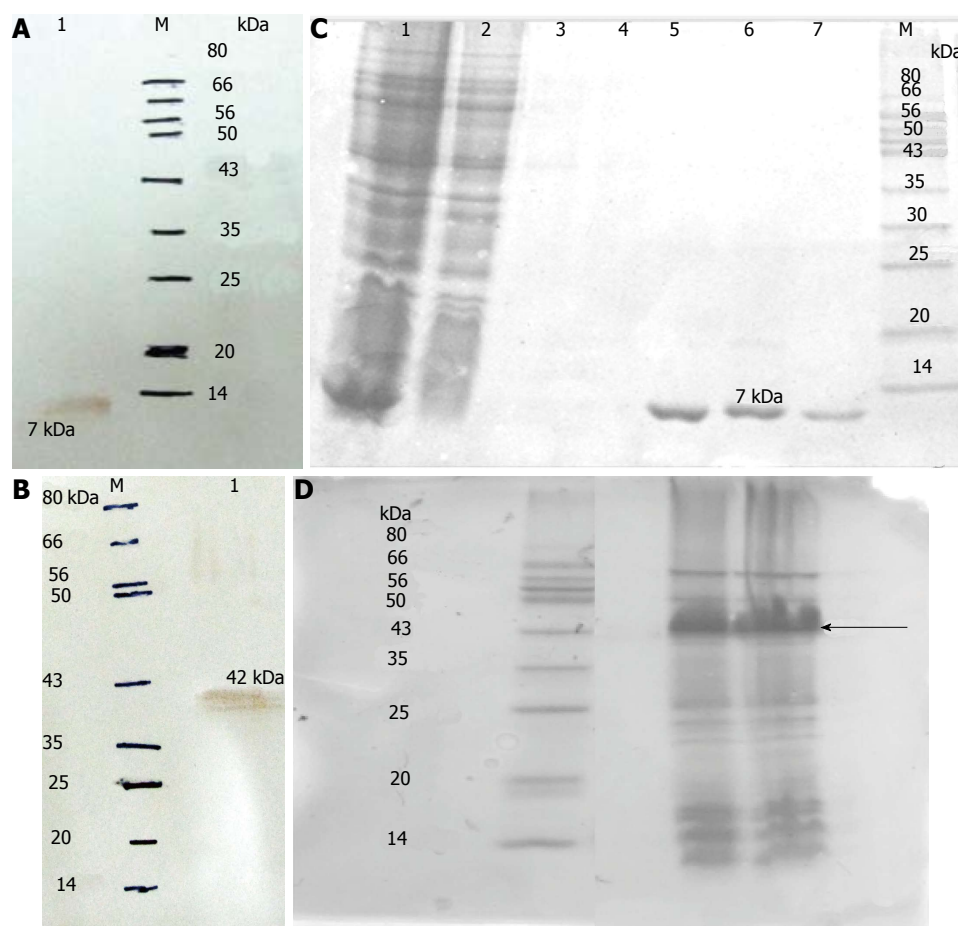


Figure 1 Western blot analysis of (A) 7 kDa recombinant N Flagellin, and (B) 42 kDa Bap subunit, (C) and (D) sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis revealing the presence of 7 kDa Flagellin and 42 kDa biofilm associated protein.

*baumannii* challenge with no mortality. Twenty percent of the mice population immunized with Bap died upon challenge with *P. aeruginosa* within 48 h. Eighty percent of the group immunized with Bap-Flagellin survived challenges with *A. baumannii* or *P. aeruginosa* (Figure 4).

#### Immunization and bacterial uptake in lungs, liver and spleen

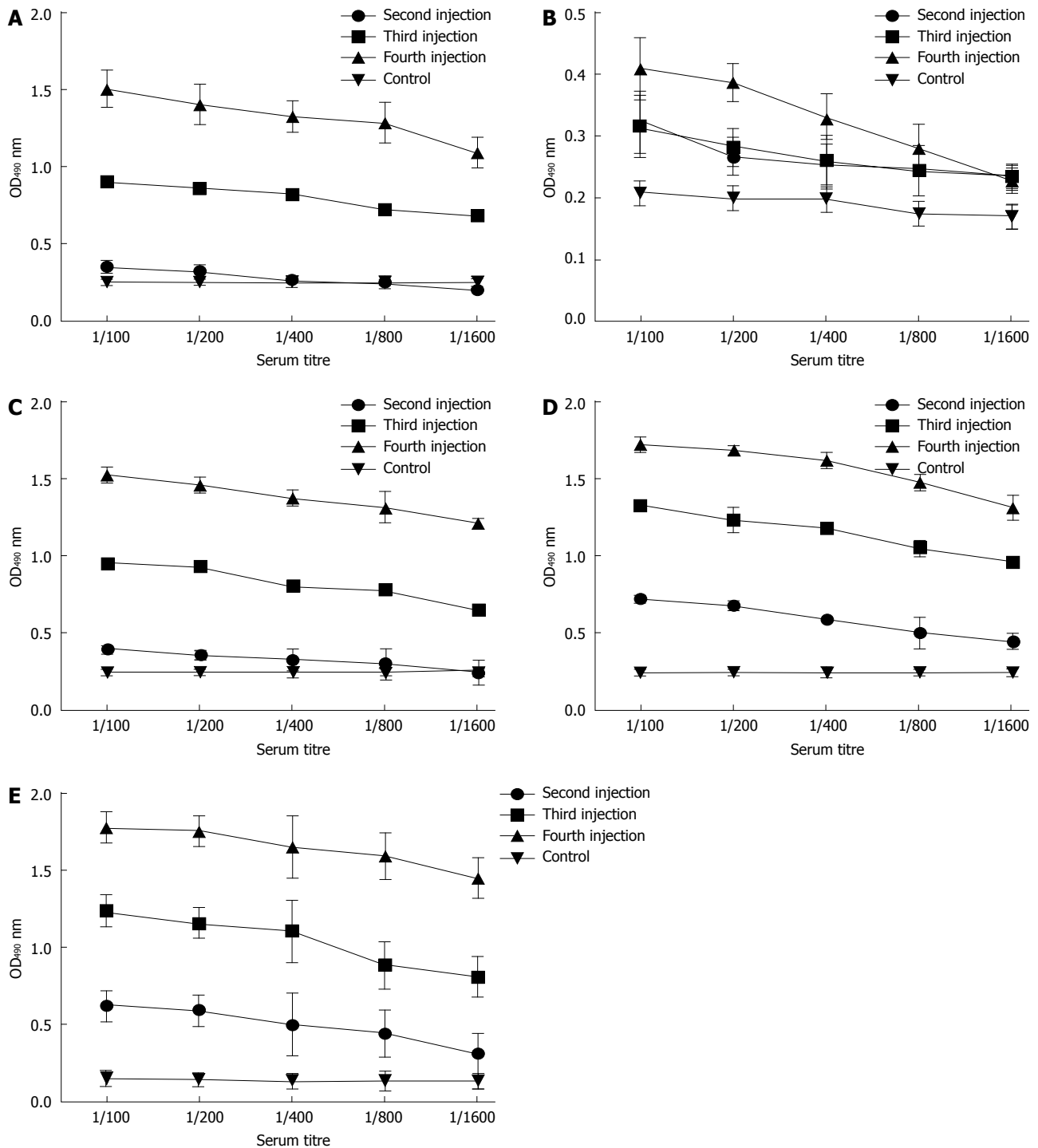
The internal organs were studied for bacterial load. *A. baumannii* infected immunized animals with Bap-Flagellin exhibited internal organs with minor bacterial load while *P. aeruginosa* PAO1 infected group showed heavy bacterial load of  $(4.3 \pm 0.12) \times 10^6$ ,  $(1.1 \pm 0.01) \times 10^6$  and  $(2.2 \pm 0.22) \times 10^6$  per gram of lungs, liver and spleen respectively. Bacterial loads in terms of CFU/g of lungs, spleen and liver of the mice group immunized with Bap were  $(1.2 \pm 0.06) \times 10^7$ ,  $(11.1 \pm 0.041) \times 10^5$  and  $(3.6 \pm 0.42) \times 10^6$  respectively. Unimmunized mice exhibited bacterial load of  $(3.20 \pm 0.11) \times 10^7$ ,  $(1.90 \pm 0.14) \times 10^6$  and  $(2.6 \pm 0.11) \times 10^6$  CFU per gram of lungs, liver and spleen respectively in challenge with *A. baumannii* and  $(2.17 \pm 0.2) \times 10^7$ ,  $(2.1 \pm 0.1) \times 10^6$  and  $(5.4 \pm 0.11) \times 10^6$  per gram of lungs, liver and spleen respectively in challenge with *P. aeruginosa* PAO1 (Figures 5 and 6).

#### Neutralization assay

Protectivity of immunized mice sera against bacterial challenge was determined by neutralization assay. There was an increased antibody titer against Bap and Bap-flagellin in the vaccinated group. As shown in Figures 4 and 5, all the experimental groups, except for Flagellin administered group were significantly ( $P < 0.05$ ) protected against *A. baumannii*.

#### DISCUSSION

Despite development of new generation antibiotics, the wide expansion of multi drug resistant *A. baumannii* is still considered as a potent threat<sup>[20]</sup>. Because of it is remarkable resistance to an extensive range of antibiotics<sup>[21]</sup>, *A. baumannii* is regarded as a problematic pathogen. Its colonization ability, survival on nutrient-limited surfaces and resistance to antimicrobial situation made it as a difficult-to-treat nosocomial pathogen<sup>[5]</sup>. Combination of biofilm forming and MDR of *A. baumannii* contribute in importance of this pathogen in Hospital - acquired infections<sup>[5]</sup>. Moreover recent researches confirm beside biofilm forming, multi drug resistance of embedded bacteria in matrix of biofilm is showed<sup>[22,23]</sup>. Using different subunits of Bap as a recombinant subunit vaccines<sup>[24]</sup>, clarify the expansion of infections caused by *A. baumannii*.

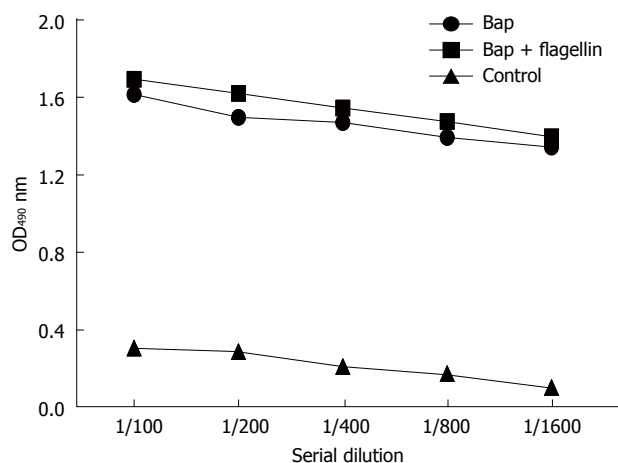


**Figure 2** Indirect enzyme linked immunosorbent assay of sera of mice injected. A: Indirect ELISA of sera of mice injected with Bap: Rise of antibody titers is significant ( $P < 0.001$ ); B: Indirect ELISA of sera of mice injected with N-flagellin subunit only: No significant rise of antibody titers was noted; C: Indirect ELISA of sera of mice injected with a single dose of Bap first followed by administration of N-flagellin in subsequent vaccination: Significant ( $P < 0.001$ ) rise of antibody titers was noted; D: Indirect ELISA of sera of mice injected with N-flagellin followed by administration of Bap in the subsequent vaccinations: Significant ( $P < 0.001$ ) rise of antibody titers was noted; E: Indirect ELISA of mice sera administered with Bap + flagellin subunit: Antibody titers increased significantly ( $P < 0.001$ ) from the second booster onwards. ELISA: Enzyme linked immunosorbent assay.

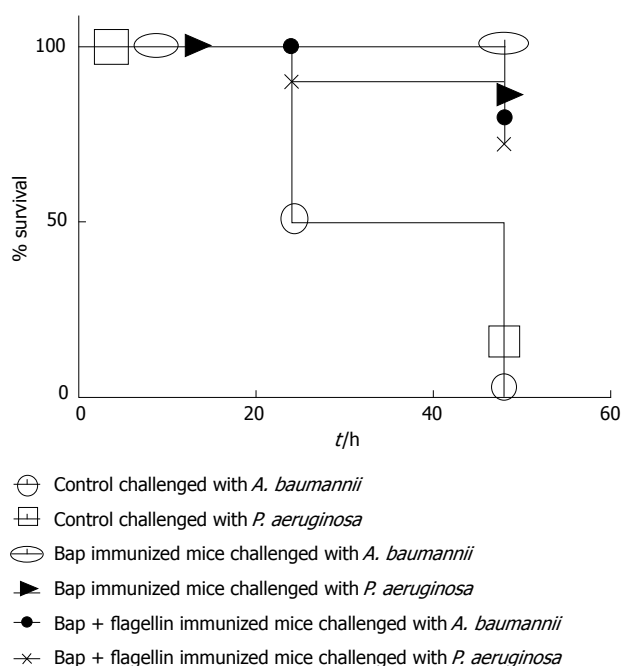
In silico studies described intercellular adhesion of *BapA* *baumannii* in maturation of biofilm<sup>[10]</sup>. Twenty or more antigenic determinants and 55 discontinuous B-Cell epitopes were predicted for Bap subunits<sup>[11]</sup>.

Adjuvant as a chemical catalyst without any considering, specific antigenic effect mount the response

to a vaccine<sup>[25]</sup>. The results (Figure 2A and D) show elevated antibody level triggered against Bap indicating that N flagellin contributed to enhancement of antigen efficacy by playing a role as an adjuvant. Physical conjugation of a vaccine antigen to a TLR ligand brings about discrimination of macromolecule from self-apoptotic bodies

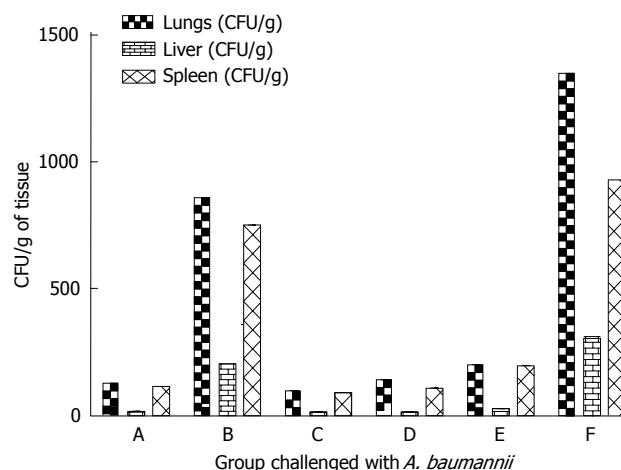


**Figure 3** Whole cell enzyme linked immunosorbent assay of mice sera immunized with Bap and Bap + flagellin against *Acinetobacter baumannii*. Significant ( $P < 0.05$ ) OD values were observed in experimental strain as compared to control group. Bap: Biofilm associated protein.

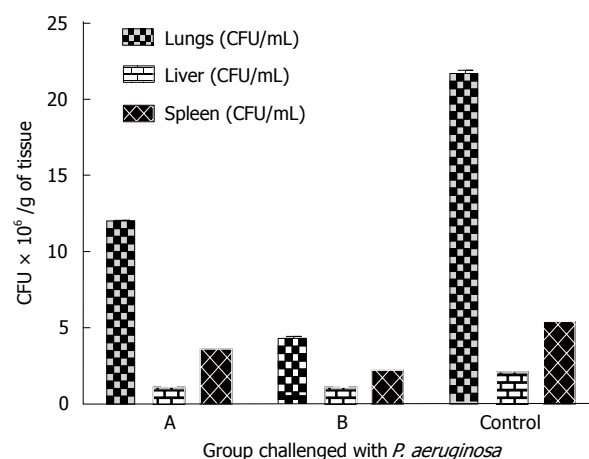


**Figure 4** Survival of mice immunized with biofilm associated protein (group A) and biofilm associated protein-flagellin (group E) challenged with *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *A. baumannii*: *Acinetobacter baumannii*; Bap: Biofilm associated protein; *P. aeruginosa*: *Pseudomonas aeruginosa*.

by the antigen presenting cells which ultimately leads to enhancement of immunogenicity and subsequently antigen presentation by MHC molecules<sup>[26]</sup>. Vance *et al*<sup>[27]</sup> investigated dissemination of *P. aeruginosa* in neutropenic mice *via* pneumonic challenge model. Bacteremia, dissemination, and eventual death of *P. aeruginosa* PAO1 challenged mice was noted upon Cy administration to the infected mice. In this work the efficacy of *A. baumannii* immunogen was studied in immunocompromised mice. Active vaccination with Bap increased survival rate and LD<sub>50</sub> of Cy-treated mice to *i.n.* challenge with *A.*



**Figure 5** Neutralization. A: The group received four doses of 20  $\mu$ g of the recombinant Bap; B: The group received four doses of 20  $\mu$ g of the recombinant N-flagellin; C: The group received a single dose of 20  $\mu$ g recombinant Bap followed by administration of N-flagellin in subsequent vaccinations; D: The group received a single dose of 20  $\mu$ g of recombinant N-flagellin followed by administration of Bap in subsequent vaccinations; E: The group received combination of Bap and N-flagellin simultaneously in all the vaccinations; F: The control group received combination of PBS and Freund's adjuvant. *A. baumannii*: *Acinetobacter baumannii*; Bap: Biofilm associated protein.



**Figure 6** Neutralization test. A: The group received four doses of 20  $\mu$ g of the recombinant Bap; B: The group received combination of Bap and N-flagellin simultaneously in all the vaccinations; control: The control group received combination of PBS and Freund's adjuvant. *P. aeruginosa*: *Pseudomonas aeruginosa*; Bap: Biofilm associated protein.

*baumannii*. Many studies described interaction of flagellin and TLR5 as a signaling of flagellin result in releasing of inflammatory mediator<sup>[28]</sup>. In silico studies describe the interaction between D1 domain of flagellin as an important region of it and TLR5<sup>[28,29]</sup>. Our study on the mice group administered only with Bap + flagellin subunit (Figure 2E) without using Freund's adjuvant showed higher antibody titer than other groups immunized with the recombinant proteins using Freund's adjuvant (Figure 2E). These results further support the adjuvant role of N-flagellin. The role of *Salmonella* Flagellin as an adjuvant has been studied. Particular interest was paid in flagellum structural subunit as an adjuvant imparting elevated immunogenicity to

soluble proteins or peptides, to activate antibody as well as cell immune responses<sup>[30]</sup>. Vaccination with chimeric flagellin provided mice with significant protection against *H. pylori*<sup>[31]</sup>. Use of EtIMP1-flagellin fusion protein has been suggested as an effective immunogen against *Eimeria* infection<sup>[32]</sup>. An experimental report on the immunogenicity of a 19 kDa merozoite surface protein-1 [MSP1(19)] from *Plasmodium vivax* C-terminal fragment against malaria and an innate immunity against the *Salmonella enterica* serovar *Typhimurium* flagellin (FlC) is available<sup>[33]</sup>. There is a report on the enhancement of FimH protective immunity against UPEC infection where the ability of FlC a Toll-like receptor 5 flagellin, a UPEC strain agonist, has been compared with Montanide ISA 206, a conventional adjuvant<sup>[34]</sup>. A TLR5 flagellin binding site at 88-97 residues located within the constant domain D1<sup>[35]</sup> coincides with that found experimentally by other researchers<sup>[28]</sup>. The importance of flagellin side chains was also described in interact with surface side chains of TLR5<sup>[35]</sup>. In this study, a 10 amino acid stretch was predicted in the N-terminal of *P. aeruginosa* PAO1 flagellin to have importance for binding to TLR5 and acting as an adjuvant<sup>[36]</sup>. Despite significant ( $P < 0.05$ ) increase in IgG titers of the mice groups immunized with Flagellin and Bap in groups C, D and E, better protection was exhibited by Bap immunized group against lethal dose of *A. baumannii* (Figure 4). Such a phenomenon could be attributed to the vaccination dose of group E which was half of the Bap quantity used in other groups. This is further supported by the absence of initial administration of Freund's adjuvant. The adjuvant role of Flagellin subunit is well documented in groups C and D where either the initial dose of Flagellin followed by the subsequent doses of Bap or initial dose of subsequently followed by administration of Flagellin subunit significantly elevated the anti Bap titer. Vaccination of mice prior to immunocompromisation and subsequent infection of mice decreases the susceptibility on the basis of lower bacterial load and better survival rate. Significant ( $P < 0.001$ ) rise in antibody titers of groups C (received 20 µg of recombinant Bap in the first vaccination followed by administration of N-flagellin in subsequent vaccinations) and D (received 20 µg of recombinant N Flagellin in the first vaccination followed by administration of Bap in subsequent vaccinations) and the 80% survival of group D challenged with either *A. baumannii* or *P. aeruginosa*, indicate the efficiency of Flagellin as an adjuvant and protective immunity against *A. baumannii*. The survival against *P. aeruginosa* could be due to the immunogenicity of the recombinant Flagellin.

Although there is no significant difference among groups A, C, D and E, higher load of bacteria was found in the liver and spleen of group E as compared to other immunized groups (Figures 5 and 6) which could explain the 20% mortality in this group (Figure 4).

In conclusion, because of the complications and difficulties in treating *A. baumannii* infections, and with respect to the significant role of Bap on one hand and the efficacy of bacterial proteins such as Flagellin as an adjuvant on the other, this model could be useful to

evaluate new vaccine regimens against *A. baumannii* infections.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

*Acinetobacter baumannii* (*A. baumannii*) is an opportunistic pathogen of major concerns. Biofilm associated protein is the most important factor in *A. baumannii* biofilm formation. Seven tandem repeats are the major functional and conserved regions components of Bap. Pattern recognition receptors, i.e., "Toll like receptors (TLRs)" are found on various microbial cells. A recombinant fusion protein strategy was used with TLR5s like Flagellin to show its role in the development of vaccine. N-terminal region amino acids of *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 flagellin was administrated to play significant binding role to TLR5. Researches show significant role of N-flagellin terminal region in immunization and can play as well as whole flagellin. The present study was designed to examine the role of *Pseudomonas* flagellin as an adjuvant to biofilm associated protein (Bap) against infections caused by *A. baumannii*.

### Research frontiers

Flagellin of *P. aeruginosa* is an important vaccine candidate. N-terminal domains are highly conserved in both type A and type B flagellins. The efficacy of gold nanoparticles (AuNPs) conjugated to N-terminal domains of *P. aeruginosa* flagellin [flagellin<sub>(1-161)</sub>], as an immunogen in mice, has been assessed by Farida. Flagellin<sub>(1-161)</sub>, AuNP-flagellin<sub>(1-161)</sub>, and flagellin<sub>(1-161)</sub> emulsified in Freund's adjuvant were administered subcutaneously to BALB/c mice. Mice given AuNP-flagellin<sub>(1-161)</sub> elicited high titers of anti-flagellin<sub>(1-161)</sub> antibodies compared with non-immune group and/or mice which received flagellin<sub>(1-161)</sub> without adjuvant. Recently, Delphine demonstrated that the adaptive responses stimulated by intranasal administration of flagellin and antigen were linked to TLR5 signaling in the lung epithelium. They sought to identify the antigen presenting cells involved in this adjuvant activity. They first found that the lung dendritic cells captured antigen very efficiently in a process independent of TLR5. However, TLR5-mediated signaling specifically enhanced the maturation of lung dendritic cells. Afterward, the number of antigen-bound and activated conventional dendritic cells (both CD11b<sup>+</sup> and CD103<sup>+</sup>) increased in the mediastinal lymph nodes in contrast to monocyte-derived dendritic cells. Their data suggested that flagellin-activated lung conventional dendritic cells migrate to the draining lymph nodes. The results demonstrated that indirect TLR5-dependent stimulation of airway conventional dendritic cells is essential to flagellin's mucosal adjuvant activity.

### Innovations and breakthroughs

In this study the authors have used a conserved region of Bap instead of the entire protein. Bap is a very large protein and its expression is near to impossible in recombinant form. There are many such proteins involved in pathogenesis of various micro-organisms. This study can be a clue to the researchers to use immunogenic conserved regions of proteins involved in pathogenesis and enhance their immunogenicity by natural adjuvants such as flagellin.

### Applications

The findings could be applied in development of novel vaccines against disease causing micro-organisms.

### Terminology

TLR: "Toll like receptors" are pattern recognition receptors present on various types of microbial cells; Bap: Biofilm associated protein. Bap *A. baumannii* is one of the biggest and the most acidic bacterial proteins with a predicted pI -3 is composed of about 8620 amino acids (aa). Seven tandem repeats are the major functional and conserved regions components of Bap.

### Peer-review

The authors present the extent which *Pseudomonas* flagellin can play a role as



an adjuvant for Bap *A. baumannii* and the model could be useful to evaluate new vaccine regimens against *A. baumannii*. This manuscript is interesting. It's just a suggestion, the N-flagellin and recombinant Bap could be fused to express.

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## Non-allergic rhinitis in children: Epidemiological aspects, pathological features, diagnostic methodology and clinical management

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### Abstract

Chronic rhinitis is a very common disease, as the prevalence in the general population resulted to be 40%. Allergic rhinitis has been considered to be the most frequent form of chronic rhinitis, as non-allergic rhinitis has been estimated to account for 25%. However, several evidences suggested that non-allergic rhinitis have been underrated, especially in children. In pediatrics, the diagnostic definition of non-allergic rhinitis has been often limited to the exclusion of an allergic sensitization. Actually, local allergic rhinitis has been often misdiagnosed as well as mixed rhinitis has not been recognized in most cases. Nasal cytology is a diagnostic procedure being suitable for routine clinical practice with children and could be a very useful tool to characterize and diagnose non-allergic rhinitis, providing important clues for epidemiological analysis and clinical management.

**Key words:** Pediatric chronic rhinitis; Non-allergic rhinitis; Nasal cytology; Local allergic rhinitis

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**Core tip:** This manuscript aims at describing the current evidences regarding non-allergic rhinitis in children, whose diagnosis is probably underrated. Here, we described the epidemiology and the diagnostic definition of non-allergic rhinitis, highlighting also the differences compared



to allergic rhinitis. Moreover, pathophysiological aspects, the emerging evidences on local allergic rhinitis and the growing role of nasal cytology in the diagnostic work-up of pediatric chronic rhinitis are discussed. Finally, insights on the therapeutic approach are provided.

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## INTRODUCTION

Rhinitis is a general term indicating any inflammatory disease of the nasal mucosa. Clinically, rhinitis is defined by the onset of two or more of the following symptoms: Nasal discharge, sneezing, nasal itching and congestion. According to the duration of nasal symptoms, rhinitis is defined as acute (resolving within 10 d) or chronic (lasting longer than 10 d). Whereas acute rhinitis is usually a viral (more often) and/or bacterial illness, isolate chronic rhinitis (without sinusitis) does not recognize infectious agents as a common etiology. Actually, the superimposition of bacterial or - less frequently - fungal infections has been more often described in the clinical setting of chronic rhino-sinusitis (CRS), however, this topic goes over the aim of this review<sup>[1]</sup>.

Although the most defined group of chronic rhinitis (CR), both in adults and children, is represented by allergic rhinitis (AR), actually those often recognize also other causes, that are attributable neither to allergic factors nor to infectious agents. Such a large portion of CR that cannot be definitively linked to allergy (and to infections) was included in a very heterogeneous "basket", named as non-allergic rhinitis (NAR): Here, several pathological entities have been enclosed and, indeed, NAR have been indicated with a number of terms (vasomotor, occupational, hormonal, atrophic, iatrogenic, idiopathic), according to the dominant clinical and/or pathologic aspects<sup>[2,3]</sup>. Thus, it is quite evident that NAR is still a very poorly defined medical entity, where several and different causes and mechanisms are supposed, and, as a consequence, it has been often misdiagnosed and/or underrated, especially in the pediatric population<sup>[4]</sup>.

Nowadays, the current definition of NAR merely relies upon the exclusion of an allergic and IgE-mediated mechanism, defining AR. Unfortunately, such a simplistic clinical distinction was complicated by the fact that AR and some forms of NAR share several clinical and pathologic features and, moreover, some patients present aspects that can be consistent with both allergic and non-allergic mechanisms, namely a "mixed"

rhinitis<sup>[5]</sup>.

## EPIDEMIOLOGY

CR is a very common disease with an increasing incidence, especially in the Western countries, where the actual prevalence in the general population is comprised between 10% and 40%, according to different studies. Despite such an epidemiological burden, CR has been considered mild disorders and that poor consideration contributed to underestimate such a diagnosis and as a consequence, the real prevalence for long time. Actually, a lot of studies focused on AR, representing the most diagnosed type of CR, showed that nasal symptoms often interfere with daily activities and alter the sleep pattern, leading to negative consequences on the social life and intellectual performances. All these aspects make CR be a global health issue characterized with significant direct and indirect costs for the society<sup>[6,7]</sup>.

In general, an allergic etiology, namely AR, can be established only in around half adult cases of CR, which indicates that a significant portion of the problem is represented by NAR. Prevalence studies on adults estimated that NAR could affect almost 20 million people in the United States, 50 million people in Europe and more than 200 million people worldwide<sup>[6,8]</sup>.

However, because of its poor clinical and pathological definition, NAR is often under-considered by clinicians and, as a consequence, the epidemiological burden is also under-estimated, especially in the pediatric age. Actually, the exact prevalence of NAR in children is not known, but the comparative prevalence between NAR and AR was estimated to be at least 1:3-4<sup>[4,9]</sup>. A recent retrospective analysis by Topal *et al*<sup>[10]</sup> regarding 472 consecutive children evaluated for CR within 1-year period, showed that 76.9% patients were diagnosed with AR and, thus, the remaining 23.1% children had NAR. Previously, another pediatric study by Chiang *et al*<sup>[11]</sup> enrolling 660 children (aged 1 to 18 year) with CR, provided very similar results, as AR was diagnosed in 75.9% cases and, by exclusion, NAR represented 24.1% of the total.

## DEFINITION AND DIAGNOSIS OF AR

AR is caused by the immunologic sensitization to one or more environmental allergens, leading to the production of specific IgE that trigger some inflammatory events responsible of the nasal symptoms. Depending upon the individual sensitization(s), AR can show different temporal patterns of symptoms, but the classical distinction between seasonal and perennial has been completed by the classification of the project Allergic Rhinitis and its Impact on Asthma (ARIA). It considers both the duration and the severity of nasal symptoms. The temporal patterns of AR have been defined by the following definitions: (1) episodic rhinitis, when nasal symptoms are very limited, as those occur if the allergic

individual comes in contact with an allergen exposure that is not a part of the daily environment (e.g., a cat at a friend's house); (2) intermittent rhinitis, if symptoms last fewer than 4 d every week or fewer than 4 wk/year; and (3) persistent rhinitis, if symptoms are present for more than 4 d a week and last more than 4 wk/year. The severity of the nasal disease is considered as being moderate to severe, if one or more of the following clinical items are reported, respectively: (1) abnormal sleep; (2) impairment of daily activities, sport and leisure; (3) difficulties at school and/or work; and (4) troublesome symptoms. If no one of these clinical aspects is present, AR is defined as being mild<sup>[12,13]</sup>.

Despite no existing study extensively described the disease-specific pattern of recurrence, duration of symptoms and impact on the quality of life of pediatric NAR, this classification could be suitable anyway and all these aspects must be evaluated in every child complaining of CR, as those are fundamental to achieve a correct diagnosis of AR or NAR. Indeed, the evidence of one or more positive results of skin prick tests and/or serum allergen specific IgE that, by itself, indicates only the allergic sensitization, must be placed into the specific clinical picture, in order to be interpreted correctly and to support a final diagnosis of AR. The diagnosis of AR to one or more environmental allergens can be safely established only if the profile of allergic sensitization(s) displayed by the individual is consistent with the temporal pattern, the persistence and the severity of nasal symptoms<sup>[12,14]</sup>. Conversely, it is not correct to reach a diagnosis to AR if nasal symptoms and the related temporal pattern, frequency and severity have not resulted to be linked to allergen exposure, although skin prick tests and/or serum of allergen specific IgE are positive for that allergen. In this case, CR is supposed not to be allergic, namely NAR, or to have more pathological components, both allergic and non-allergic, namely mixed rhinitis or overlapped rhinitis, as discussed forward. In conclusion, the nasal anatomy must be always considered too in the diagnostic pathway of CR, as anomalies of turbinates and septum can contribute to the nasal disease; moreover, adenoid hypertrophy must be investigated in children. Finally, the occurrence of superimposed chronic rhinosinusitis (CRS) should be actively excluded in the setting of AR with atypical symptoms and/or when the clinical disease and the pattern of environmental sensitization seem to be mismatching<sup>[15]</sup>.

## NAR

Currently, NAR is basically diagnosed by exclusion of AR in the appropriate clinical setting. NAR is a chronic condition of the nasal mucosa showing symptoms of nasal congestion and rhinorrhea with no evidence of allergic sensitization through skin prick tests (SPT) and dosage of serum specific IgE for environmental allergens<sup>[16]</sup>.

According to such a definition, it is estimated that

at least a quarter of patients complaining of CR have NAR, but they might be more numerous, considering that some have mixed rhinitis and that NAR are still underrated<sup>[17]</sup>. According to data from United States National Rhinitis Classification Task Force, which were collected more than 15 years ago, around 17 millions of Americans were affected with NAR and as many people suffered with a combination of non-allergic and allergic nasal disease (mixed rhinitis). As a percentage, AR, NAR and mixed rhinitis affected 43%, 23% and 34% of patients, respectively. Moreover, it was evident that NAR and mixed rhinitis occurred more frequently in adults than in children, were more common in female patients and used to have a perennial rather than seasonal course<sup>[18,19]</sup>. These observations have been replicated by many other studies and the prevalence of NAR ranged between 17% to more than 50%<sup>[20-22]</sup>.

Although NAR were considered to be more prevalent in adults, actually those could represent a significant burden in the pediatric age, too. Unfortunately, detailed information regarding the prevalence and burden of NAR in children is lacking: That may partially be explained by the few allergen challenges that are performed in young ages<sup>[21]</sup>. Moreover, the term NAR does not indicate a specific clinical entity and, therefore, includes a number of different forms of CR: As a consequence, several classifications and terminologies generated further imprecisions in the epidemiological evaluations. Indeed, the classification of NAR still relies upon the presence of comorbidities and/or the evidence of triggering factors and/or some pathological features. Unfortunately, most cases of NAR have not been associated to any of the aforementioned conditions and, thus, have been defined as being idiopathic and/or vasomotor<sup>[3]</sup>.

According to the Global Atlas of Allergic Rhinitis and Chronic Rhinosinusitis edited by the European Association of Allergy and Clinical Immunology (EAACI)<sup>[23]</sup>, the following forms of NAR have been recognized: (1) non-allergic rhinitis with eosinophilia syndrome (NARES); (2) hormonal Rhinitis (pregnancy, associated to menstrual cycle, acromegaly, hypothyroidism); (3) rhinitis of the elderly; (4) gustatory rhinitis (hot and spicy foods, alcohol consumption, etc.); (5) atrophic rhinitis (primary or secondary to sinus surgery, autoimmune and/or immune-mediated diseases); (6) cold-air induced Rhinitis (triggered by cold and/or windy climate conditions); (7) drug-induced Rhinitis (nasal decongestant "rhinitis medicamentosa"), aspirin, systemic alpha- and beta-adrenergic antagonist, phosphodiesterase inhibitors, calcium channel blockers, neuroleptics, etc.); (8) occupational non-allergic rhinitis (irritants, corrosive substances); and (9) idiopathic rhinitis ("vasomotor rhinitis").

The NAR consensus panel of World Allergy Organization (WAO) edited a similar classification, excluding automatically both anatomical/mechanical nose abnormalities and CRS. Moreover, systemic medical conditions (endocrine/metabolic, autoimmune and miscellanea) leading to NAR symptoms have been

**Table 1** Classifications of non-allergic rhinitis by World Allergy Organization and European academy of allergy and clinical immunology<sup>[23,24]</sup>

WAO	EAACI
Drug-induced rhinitis	Drug-induced rhinitis
Local $\alpha$ -adrenergic agonists ("rhinitis medicamentosa": Excessive use of nasal decongestants); systemic $\alpha$ - and $\beta$ -antagonists; aspirin; phosphodiesterase (PDE) V inhibitors; ACE inhibitors; calcium channel blockers; antipsychotics	
Gustatory rhinitis	Gustatory rhinitis
Anterior rhinorrhea and/or post-nasal drip after eating, especially hot or spicy foods	
Hormonal-induced rhinitis	Hormonal rhinitis
Rhinitis of pregnancy and menstrual cycle-associated rhinitis	
NARES	NARES
Presence of eosinophilia in the nasal secretions	
Occupational rhinitis	Occupational non-allergic rhinitis
Irritant-induced rhinitis and corrosive rhinitis	
Senile rhinitis	Rhinitis of the elderly
Persistent watery rhinorrhea without any identifiable trigger	
Atrophic rhinitis	Atrophic rhinitis
Primary or secondary (extensive surgery, chronic granulomatous disorders, other)	
Non-allergic rhinopathy	Idiopathic rhinitis
Nasal congestion and/or rhinorrhea triggered by irritants and/or weather changes, but also chronic without identifiable triggers	
Cerebral spinal fluid leak	Cold air-induced rhinitis
Persistent rhinorrhea after	Rhinorrhea and/or nasal congestion
cranio-facial trauma or	and/or burning triggered by cold and/or
facial/sinus surgery	windy condition

NARES: Non-allergic rhinitis with eosinophilia syndrome; WAO: World Allergy Organization; EAACI: European academy of allergy and clinical immunology.

considered separately. Thus, eight subtypes of NAR have been classified: (1) drug-induced rhinitis; (2) gustatory rhinitis; (3) hormonal-induced rhinitis (including responses to endogenous female hormones, basically the rhinitis of pregnancy); (4) non-allergic rhinitis with eosinophilia; (5) senile rhinitis; (6) atrophic rhinitis; (7) cerebral spinal fluid leak; and (8) non-allergic rhinopathy (corresponding to vasomotor rhinitis and those forms related to climate conditions)<sup>[24]</sup>.

Taking in account what stated above, it is evident that a systematic classification of NAR based upon pathological mechanisms is still distant (Table 1). Therefore, rather than trying to find some correspondences of the aforementioned categories of NAR in the pediatric population, it seems to be more useful to discuss some specific aspects of NAR, such as the emerging evidence on local allergic rhinitis (LAR), the growing interest on nasal cytology for the possibility of defining the features of NAR inflammation and the pathogenic role of some noxious agents for the nose, such as environmental pollutants.

## LAR

LAR is a type of CR showing the same phenotypic characteristics as AR, actually without any remarkable positivity of environmental skin-prick tests (SPT) and/or

serum specific IgE. In specific studies, LAR is characterized by an allergen-related nasal hyper-reactivity, despite the absence of specific systemic atopy, as could be evidenced though specific nasal provocation tests (NPT), performed by administering intra-nasally a set of purified airborne allergens. In this clinical setting, the positive response to specific NPT suggested the presence LAR, which could be confirmed by recovering also allergen-specific IgE in the nasal mucosa<sup>[25,26]</sup>. Indeed, LAR and AR have been demonstrated to have similar patterns of inflammation, sustained by Th2 polarized immune responses: Probably, different initiating immunological events and mechanisms converging to a final common pathway of nasal inflammation may exist<sup>[27,28]</sup>.

The local production of IgE in the nasal mucosa has been largely demonstrated in patients with AR, where the allergen exposure directly drives the antibody class switch recombination<sup>[29,30]</sup>. The presence of IgE specific to house dust mite in the nasal mucosa of patients with CR displaying negative SPT, but positive specific NPT, was reported in 1975 by Huggins *et al.*<sup>[31]</sup>. Starting from 2000, Carney and Powe published a series of studies investigating nasal allergy due to the local production of IgE, defined as "entopy". They were able to demonstrate the presence of mast cells, eosinophils and IgE+ cells in a selection of archival samples of nasal mucosa from patients affected with idiopathic CR: In addition to an increased number of mast cells, some showed a positive staining for IgE specific to grass pollen<sup>[32]</sup>. Eventually, Rondón *et al.*<sup>[33]</sup> and Wise *et al.*<sup>[34]</sup> described a cohort of NAR patients manifesting positive response to specific NPT to house dust mite: That result was reported in around 50% cases and, interestingly, specific IgE were detected in the nasal lavage of more than 10% of those.

Poor SPT technique and/or quality of allergen preparations or a cover allergy, namely the sensitivity to not tested allergens, have been suggested to explain some cases of apparent entopy. Additional pathological mechanisms, such as non-IgE mediated hypersensitivity and other tissue-specific immune responses could be considered in patients showing specific nasal hyper-reactivity, but not local IgE<sup>[35]</sup>.

Epidemiologically, LAR might affect more than 40% people diagnosed with NAR currently. In a study including 428 patients with chronic rhinitis, Rondón *et al.*<sup>[36]</sup> diagnosed AR, LAR and NAR in 63%, 26% and 11% of patients, respectively. Importantly, in addition to highlighting the epidemiological importance of LAR among chronic rhinitis, this study noticed that 36% of patients with LAR had rhinitis since childhood. Moreover, some authors suggested that LAR might be the first step of the natural history of AR, especially in polysensitized and young patients<sup>[37]</sup>. Indeed, some studies showed that children could develop systemic atopy to grass pollen only in the second or third season of nasal symptoms<sup>[38]</sup>. However, such a hypothesis upon LAR as an initial stage of AR needs to be tested adequately and immune processes might differ according to allergens. So far, the most prevalent allergen sensitizations identified in

patients with LAR have been house dust mite, grass and olive pollen, but other allergens, including molds, animal dander and occupational substances, have not been completely investigated yet and might have a role<sup>[39,40]</sup>.

However, that LAR is not only an adult disease and can arise from the pediatric age was evident also in the large follow-up study by Rondón *et al.*<sup>[41,42]</sup> at least 35% of patients diagnosed with LAR and followed-up were 14-20 years old, confirming that the diagnosis should be considered in children too. Actually, this study showed also that the rate of conversion of LAR to "systemic" AR, namely displaying positive SPT and/or serum specific IgE, was only 6.8% and was similar to the percentage observed in healthy controls. Thus, these observations seemed to support the concept that LAR and AR could be different pathological entities. The first pediatric study assessing specific nasal hyper-reactivity and mucosal IgE was carried out by Fuiano *et al.*<sup>[43]</sup> who analyzed 192 children aged 3-15 years and showing at least one positive SPT for aeroallergens (among house dust mite, grass pollen, olive pollen, *Parietaria* and *Alternaria*): Of those, 67.6% were symptomatic and 34.6% were asymptomatic. Between these two groups, a striking difference in nasal IgE was found, being 77% vs 13%, respectively. This study suggested a major role of nasal IgE in determining symptoms in children sensitized to environmental allergen, but also highlighted the presence of other mechanisms than or in addition to the production mucosal IgE, being able to suppress its activity (in asymptomatic children positive for nasal IgE) or to replace it (in symptomatic children negative for nasal IgE)<sup>[44]</sup>. The same authors replicated similar analysis in children suffering from chronic rhinitis during the period when *Alternaria* spores can be present in the environment. Interestingly, they found that most children (64.3%) had negative SPT for *Alternaria*, but were positive for nasal specific IgE; only 16.1% were positive to both tests and the remaining 19.6% had a positive SPT without nasal IgE. These results represented the first pediatric evidence that an allergic sensitization manifesting with chronic rhinitis can be mediated by an exclusive production of specific IgE in the nasal mucosa. Unfortunately, current evidences on pediatric LAR are insufficient to draw any consistent conclusion, as appropriate prospective studies are still lacking<sup>[45]</sup>.

## PATHOPHYSIOLOGICAL ASPECTS

According to the findings of studies including nasal cytology, AR can display different forms and severity of inflammation according to the allergic sensitization and, possibly, the season. Similarly, NAR can be classified through several inflammatory patterns. These observations supported the concept that several immune-pathological mechanisms could be involved in both AR and NAR. Moreover, nasal cytology also demonstrated that AR and NAR coexist in some patients, leading to so-called overlapped rhinitis: Thus, more mechanisms seem

to interplay or, perhaps, some of those could be shared between AR and NAR<sup>[46]</sup>. Importantly, some studies showed synergistic interactions in the inflammatory nasal responses between the specific IgE-mediated component of AR and the superimposition of non-specific irritation induced by environmental pollutants (e.g., diesel exhaust particulate, ozone, etc.), maybe in a bidirectional way<sup>[5]</sup>.

By definition, AR and LAR are sustained by an IgE-mediated inflammation and are mainly associated to a Th2 immune response. However, among seasonal and perennial forms of AR, nasal cytology evidenced different features of the inflammatory infiltrate into the nasal mucosa, which suggested some substantial differences in the pathophysiological immune cascade anyway. On the contrary, by exclusion, NAR included all that is not promoted by an IgE-mediated pathogenesis. A series of non IgE-mediate immune responses are plausible, considering the heterogeneity of NAR, as evidenced by the nasal cytology and by the clinical observation. Unfortunately, most pathophysiological aspects of NAR have not been unveiled yet. Moreover, in addition to immune-mediated mechanisms, several evidences showed that some neurogenic responses seem to play a fundamental role in the development of NAR inflammation and, probably, are involved in AR, too<sup>[47-49]</sup>.

Non IgE-mediated immune mechanisms could take place in several classified forms of NAR. In occupational rhinitis, lymphocytic infiltrates have been described, in association to epithelial desquamation and glandular hypertrophy. In atrophic rhinitis, different inflammation patterns can be seen, including granulomatous lesions. Conversely, inflammatory changes are less evident in other forms of NAR: For instance, in gustatory rhinitis, rhinitis medicamentosa or idiopathic (vasomotor) rhinitis, the nasal symptoms mainly resulted from the increased glandular secretion and the mucosal edema due to a local transudate, rather than from the presence of exudate and abundant inflammatory cells. Indeed, some authors preferred to refer these forms as rhinopathy, rather than rhinitis<sup>[3,24,50]</sup>.

Therefore, in addition to pure immunologic mechanisms, actually a complex series of neuroendocrine pathways have been proposed to explain the pathophysiology of several forms of NAR and, in some extent, those could be involved in AR too. Probably, both immune and neuroendocrine systems interact in the nasal mucosa of subjects suffering from chronic rhinitis, but those might have a different importance according to the type of NAR. Some evidences supported the involvement of autonomic neural responses in the pathophysiology of NAR. The neural regulation of upper airways relies upon sympathetic (adrenergic) and parasympathetic (cholinergic) fibers, which regulate the activity and the trophism of epithelial, vascular and glandular components of the nasal mucosa. Sympathetic neuromediators, being mainly norepinephrine and neuropeptide Y, cause local vascular constriction; however, in a lesser extent, those innervate also the glandular structures, decreasing the nasal secretions. Conversely, parasympathetic fibers, through the secretion



of acetylcholine and some neuropeptides (particularly, vasoactive intestinal peptide), stimulate nasal glands and induce vasodilation. Actually, the neural system of the nasal mucosa includes also the sensory innervation supplying the septum, the lateral walls, the anterior parts of nasal floor and the inferior meatus. Among these nerves, non-adrenergic and non-cholinergic nervous fibers have been demonstrated in the human nasal mucosa. Predominantly, those resulted to be sensory unmyelinated C-fibers and have been demonstrated to be involved in the realization of several protective nasal responses (such as sneezing, mucus production and mucosal congestion) against potential noxious stimuli entering in the nasal cavities. The activation of these unmyelinated sensory C-fibers leads to the release of several neuropeptides [Substance P (SP), calcitonin gene related peptide (CGRP) and neurokinins] in the human nasal mucosa, through an antidromic conduction, in response to a large variety of stimuli<sup>[50,51]</sup>.

Thus, this neurogenic reactivity seems to be dominant in those forms of NAR where the cellular inflammation resulted to be poorly expressed. However, some evidences suggested that the neural factors could play a role also in AR and in other forms of NAR anyway. Interestingly, a denser innervation of sensory C-fibers in the nasal mucosa was demonstrated in different forms of NAR, including idiopathic rhinitis, occupational rhinitis and some drug-induced rhinitis. Moreover, this hyper-innervation was associated to an increased expression of some neuropeptides (SP and CGRP, in particular) in the mucosal nerve fibers and, importantly, this finding was observed also in patients with AR too, where some neuroinflammatory mechanisms might worsen the clinical expression of AR and/or promote the occurrence of overlapped rhinitis. Indeed, further investigations might determine whether overlapped rhinitis could be part of a continuum between AR and NAR, where immunologic responses (both IgE-mediated and non IgE-mediated), inflammatory/irritant responses upon exposure to chemicals and/or particulate matter and neurogenic factors develop over the time starting from an initial trigger<sup>[52-54]</sup>. In fact, non specific nasal hyper-reactivity, being an abnormal or excessive reaction of the nasal tissue (in term of glandular activity, mucosal inflammation and vascular leakage) after the exposure to a non allergenic stimulus that is usually innocuous to most people, have been demonstrated in patients with both NAR and AR. Such a nasal hyper-reactivity seemed to result mainly from the impaired balance of the activity of the aforementioned neural local reflexes. Indeed, several inflammatory mediators have been demonstrated to interact with sensory nerve endings in the nasal mucosa, which resulted to release several neurotrophins, in addition to the aforementioned neuropeptides. The formers would be responsible of the hyper-innervation seen in chronic rhinitis, both AR and NAR, whereas the latters would contribute to up-regulate the local inflammation by promoting the transcription of pro-inflammatory cytokines, in addition to eliciting

the vascular and glandular responses causing nasal symptoms<sup>[55,56]</sup>.

## DIAGNOSIS OF NAR IN CHILDREN

Pediatric NAR is poorly defined: The available information is quite heterogeneous and most concepts have been derived from NAR in adults. Indeed, there are no pediatric studies recognizing the categories of NAR, as classified by EAACI or WAO, as those classifications were tailored for the adult population: By instance, hormonal, atrophic and occupational rhinitis are related to adult diseases or activities, as well as drugs involved in the occurrence of NAR are not used in the pediatric age and food causing gustatory rhinitis are not usually given to children. Therefore, the current diagnostic work-up of pediatric CR is often limited to the identification of AR, NAR (in general) and mixed rhinitis<sup>[4,21]</sup>. Such a diagnostic limitation is partially due to the fact that investigational techniques being useful to evaluate nasal obstruction (e.g., rhinomanometry) and nasal hyper-reactivity (e.g., nasal provocation test) cannot be applied to children so easily as to adult people, where those have been considered as important diagnostic tools, in order to define objectively the entity and the trigger factors of chronic rhinitis<sup>[57]</sup>.

Rhinomanometry is a technique allowing an objective estimation of nasal airway obstruction and, therefore, should be also an essential part in the setting of nasal challenge procedures<sup>[58]</sup>. Whereas some nasal symptoms, like rhinorrhea and sneezing, are clinically evident, actually nasal blockage could be difficult to be assessed subjectively: The individual perception of nasal obstruction can be influenced by several factors and could mismatch with objective measurements of nasal patency. Indeed, available pediatric studies on the correlation between subjective scores and objective techniques for estimating nasal obstruction provided conflicting results<sup>[59,60]</sup>. Recently, a pediatric study including 284 children aged 6-14 years showed that a major part of children under-estimate or over-estimate their nasal obstruction and concluded that an objective measurement of nasal patency could improve the clinical management<sup>[61]</sup>. Here, children were evaluated by anterior active rhinomanometry that provides an accurate evaluation of nasal obstruction or resistance through the measurement of nasal airflow generated by a known pressure gradient<sup>[61,62]</sup>. An alternative technique is represented by acoustic rhinometry, which is basically based upon the amplitude and temporal analysis of the reflections of incident sound waves on the nasal cavities, calculating the cross-sectional area at different depths<sup>[63]</sup>. However, both techniques require considerable standardization to obtain reproducible results and experience to interpret those. Actually, Peak Nasal Inspiratory Flow could represent a simple, reproducible and inexpensive technique, but it is effort dependent and, thus, could be difficult to apply to the pediatric population<sup>[57]</sup>.

Another important tool in the differential diagnosis of NAR should be the nasal provocation test (NPT) and, ideally, the objective measurement of nasal obstruction should be used to assess the results, in addition to symptom scores. Specific NPT consists of triggering a nasal allergic response by administering a standardized allergenic extract locally in the nose. Non Specific NPTs have been used to evidence non-allergic nasal hyper-reactivity, namely an abnormal nasal reaction upon the exposure to several physical (e.g., cold air) or chemical (e.g., methacholine, mannitol, distilled water, etc.) agents being innocuous for most people. However, their use has been often limited to the research field and more studies are needed to evaluate the clinical usefulness and to standardize the methodology<sup>[56,58]</sup>.

Specific NPT was primarily introduced in order to evaluate the clinical significance of a specific allergen sensitization in multi-sensitized patients. In the clinical setting of NAR, specific NPT could be useful to diagnose LAR, through the positive response to specific allergens in the setting of a chronic rhinitis without evidence of systemic atopy. Unfortunately, as discussed above, both techniques for estimating nasal patency objectively and NPT are difficult to be used in the routine allergy practice in children<sup>[64,65]</sup>. Indeed, the daily clinical experience in the pediatric allergy clinic includes children showing a clinical history consistent with AR in absence of positive findings at SPT and/or serum specific IgE. Here, the potential alternative to the complicated execution of NPT and/or rhinomanometry might be the detection of allergen specific IgE in the nasal mucosa<sup>[45]</sup>. Marcucci *et al.*<sup>[66]</sup> described a method to detect nasal IgE in children, by placing into the nostril (in contact with the septum nasal mucosa) a paper strip whose surface had been covalently coupled with a specific allergen. Recently, Fuiano *et al.*<sup>[44]</sup> recalled this method and carried out some pediatric studies by using nasal strips where a colorimetric reaction provided a semi-quantitative evaluation of the presence of specific nasal IgE. The authors sought nasal IgE specific to some aeroallergens (*Alternaria*, house dust mites, Grass Pollen, Olive pollen and *Parietaria*) in 192 children with positive SPT for one or more aeroallergens. A significant association between the presence of specific nasal IgE and nasal symptoms was observed in systemically sensitized children. Eventually, another study showed that measuring nasal IgE could be useful to unveil local allergic rhinitis to *Alternaria* and to avoid a misdiagnosis of NAR<sup>[66]</sup>. Alternatively, specific nasal IgE have been measured in the nasal secretions by immunoassay: Rondón *et al.*<sup>[67]</sup> described a detection rate of specific nasal IgE to grass pollen of around 30%-35% in their cohort of LAR patients (defined by the positivity to specific NPT), which suggested a limited sensitivity of their method or perhaps the involvement of other immunologic mechanisms.

In summary, the testing of nasal specific IgE still needs to be improved and standardized and there are no available studies regarding the diagnosis of LAR in children through the evaluation of nasal IgE and/or

specific NPT<sup>[45]</sup>. Recently, Gómez *et al.*<sup>[68]</sup> proposed a role for basophil activation test (BAT) in the diagnosis of LAR: They found a sensitivity of around 50% in patients diagnosed with LAR to house dust mite, but that remains an isolated experience till now.

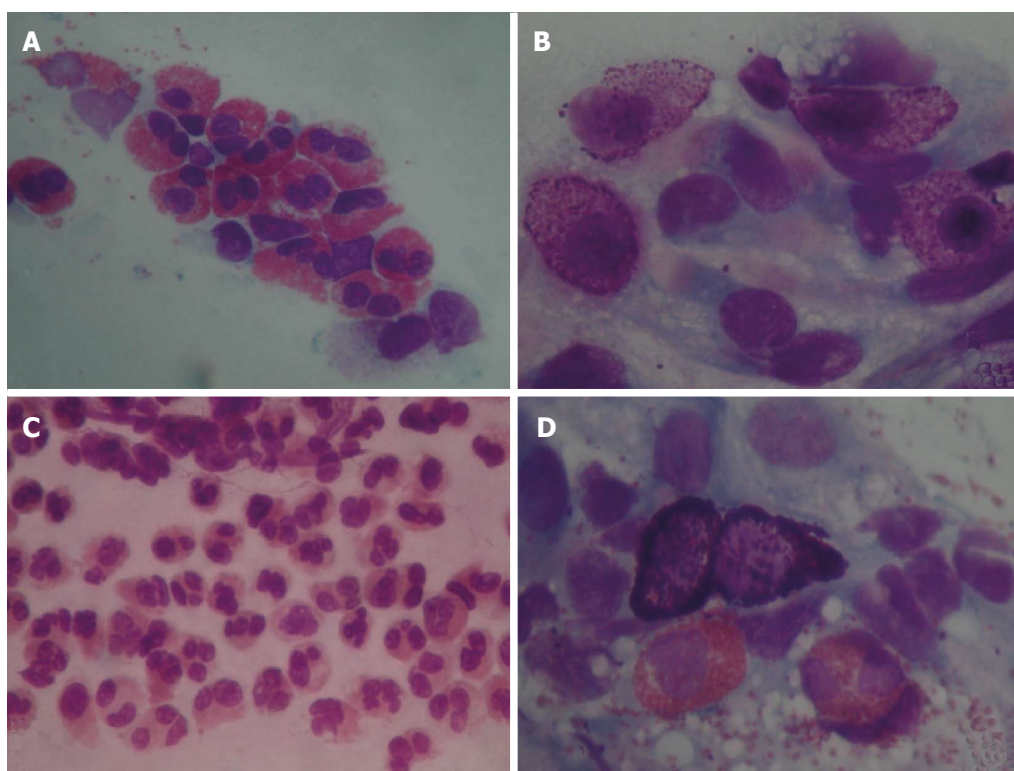
Thus, the diagnostic work-up of NAR in children is still limited to its definition by exclusion of AR in most cases, as further diagnostic investigations cannot routinely performed in children (e.g., specific NPT, rhinomanometry, acoustic rhinometry) or showed no acceptable sensitivity and/or standardization (nasal specific IgE, BAT). However, a simple and inexpensive method to investigate patients (including children) with chronic rhinitis is represented by nasal cytology that might allow the description and classification of nasal diseases according to the inflammatory features. Moreover, a number of studies regarding nasal cytology have been performed also in the pediatric age too and, therefore, a specific section will be dedicated.

## NASAL CYTOLOGY

Nasal cytology consists of the microscopic analysis of surface cells of nasal mucosa. The suitable biological sample can be collected through a sterile swab or by scraping and is usually obtained by anterior rhinoscopy in order to reach the middle portion of the inferior turbinate. Thus, the material must be placed on a glass slide and, after it is fixed by air-drying, it can be stained according to May-Grunwald-Giemsa method. This simple staining allows identifying correctly all the normal cellular components of the nasal mucosa, the inflammatory cells and also bacteria and fungi, if those are present. Therefore, nasal cytology is endowed with several aspects allowing a routine use in allergy daily practice, including the pediatric setting: It is non-invasive, easy to perform, non-time-consuming and inexpensive<sup>[69]</sup>.

The result of nasal cytology is the rhinocytogram that, in healthy controls, shows only ciliated cells and mucous-secreting cells of the pseudo-stratified epithelium of the nasal mucosa, in addition to sparse neutrophils. On the contrary, the presence of more abundant neutrophils and/or other inflammatory cells (eosinophils, mast cells, lymphocytes), as well as the presence of bacteria and fungi, represents a sign of nasal pathology. Nasal cytopathology has been studied in several nasal diseases and it might be a promising diagnostic tool for allergic and non-allergic rhinitis<sup>[70]</sup>.

AR can be caused by several environmental allergens, differing as regards the immunological properties and the presence during the year. AR caused by indoor allergens, such as house dust mite, are usually perennial, as the patients complain of nasal symptoms all over the year and, accordingly, the rhinocytogram is characterized with a persistent infiltration of neutrophils and, in a lesser extent, of eosinophils, describing a pathological pattern of "minimal persistent inflammation". Seasonal AR is often triggered when the pollens are present in the atmosphere and, therefore, the nasal symptoms are



**Figure 1 Nasal cytology of non-allergic rhinitis.** A: NARES; B: NARMA; C: NARNE; D: NARESMA. NARES: Non-allergic rhinitis with eosinophilia syndrome; NARMA: NAR with mast cells; NARNE: NAR with neutrophils; NARESMA: NAR with eosinophils and mast cells.

limited within a specific period of the year. In this setting, the rhinocytogram is very rich of all inflammatory cells, including neutrophils, lymphocytes, eosinophils and mast cells; by contrast, the cytological aspect of nasal mucosa can be completely normal outside the pollen season<sup>[71]</sup>.

Interestingly, nasal cytology provided a heterogeneous landscape of non-allergic rhinitis, both in adults and children, but EAACI and WAO classifications considered only non-allergic rhinitis with eosinophilia syndrome (NARES), as a separated cyto-pathological entity of NAR<sup>[3]</sup>. That should include those patients having the same clinical features as AR, but not positive SPT and/or serum specific IgE, namely LAR<sup>[72]</sup>. Under this limited perspective, all the other variants of NAR could be defined as non-NARES. Actually, nasal cytology suggested that the situation is more complex than two variants of NAR<sup>[73]</sup>; moreover, very recent evidences suggested that also NARES might recognize immunological mechanisms other than entropy<sup>[74]</sup>. Gelardi *et al.*<sup>[73]</sup> described at least four cytological patterns of NAR (Figure 1): (1) NAR with eosinophils (NARES); (2) NAR with mast cells (NARMA); (3) NAR with neutrophils (NARNE); and (4) NAR with eosinophils and mast cells (NARESMA).

These authors tried defining the incidence, the clinical aspects and the prognosis of these different cytological forms of “cell-mediated non-allergic rhinitis”. NARES and NARNE resulted to be the most abundant forms among children, as those were recognized in 46.5% and 40.6% cases, respectively. NARMA has been detected in 10.5%

cases and NARESMA in the remaining 2.6%. On the contrary, in adulthood the relative proportions among all these forms of NAR seem to be more balanced and, particularly, NARESMA has been reported as affecting around 25%-30%, like NARES. Importantly, these two types resulted to be associated to the most severe clinical manifestations, such as non-allergic asthma, nasal polyposis and aspirin intolerance. Particularly, NARESMA was described as leading to the worst complications, including major respiratory disturbances (sleep-apnea and severe asthma). Significant respiratory symptoms and complications were almost absent in children with NARMA: Thus, the presence of eosinophils in the nasal mucosa resulted to be most important determinant for the severity of the clinical disease. Despite the significant portion of NARES in the pediatric population, actually respiratory severe symptoms have not been seen as in adults, as those probably require a number of years of rhinopathy to develop. The other prevalent form of NAR in children resulted to be NARNE, as described above. NARNE was associated to cystic fibrosis, but it is supposed to have a multifactorial etiology and, particularly, it has been linked to the exposure to a number of irritants, including air pollution and cigarette smoke<sup>[73,75-77]</sup>.

A large series of pollutants and chemicals have been reported to be involved in the etiopathogenesis of “environmental” NAR. Many of those have been related to occupational exposures (*e.g.*, volatile organic compounds, paper dust, acetic acid, *etc.*), but

others became common environmental pollutants<sup>[78]</sup>. A significant difference of inflammatory changes in the nasal cytology specimens was observed between people living in highly polluted urban areas and rural residents<sup>[79]</sup>.

Environmental tobacco smoke, including sidestream or second-hand tobacco smoke exposure, has been demonstrated to have detrimental effect on several organs, especially in children. A wide variety of tobacco smoke effects have been described on the immune system and, of course, respiratory airways - and nose as first - are particularly exposed. Effects on immune cells functions and cytokines production have been reported in nasal mucosa and adenoidal tissue of children exposed to passive smoke<sup>[80]</sup>. Another important irritant for the respiratory system is ozone, whose concentration in the ground-level atmosphere increases in polluted areas. Experimental studies showed that ozone induces epithelial abnormalities and inflammatory responses of the nasal mucosa. A pediatric study reported a significant association between outdoor ozone concentration and levels of leukocytes in the nasal secretions<sup>[81]</sup>. Similarly, the particulate matter of the urban environment and, particularly, particles derived from diesel exhaust, have been described to increase the expression of inflammatory cells and molecules at several level of the respiratory system, including the nasal mucosa. Also fine particulate matter ( $PM \leq 2.5 \mu m$ ) was shown to increase significantly the percentage of eosinophils and several inflammatory mediators in the nasal lavage fluid of asthmatic children, but not in healthy children<sup>[82]</sup>. Thus, environmental pollution could exacerbate allergic inflammation and/or promote the development of new allergic sensitization at the respiratory level, especially in children, leading to mixed patterns of allergic and non allergic rhinitis in some cases<sup>[83,84]</sup>.

Nasal cytology provided another important contribution under this perspective, as introduced the concept of mixed of "overlapped" rhinitis, namely the simultaneous presence of different forms of inflammatory rhinopathies in the same patient<sup>[85]</sup>. Particularly, there are patients (including children) diagnosed with AR, but that diagnosis actually is not fully consistent with the nasal symptoms and/or the related temporal pattern. In these cases, including both seasonal and perennial forms of AR, the superimposition of a form of NAR could be suspected and nasal cytology could provide fundamental clues. By instance, there are children suffering with a pollen-related AR who have nasal symptoms during the winter, when the rhinocytogram can show the presence of eosinophils, as well as there are children with house dust mite AR who have an abnormal clinical course and whose rhinocytogram can display mast cells and/or eosinophils, in addition to the pattern of minimal persistent inflammation (showing usually a preponderance of neutrophils). Those are typical examples of patients where an overlapped rhinitis should be suspected. Gelardi M and Landi M proposed some clinical and cytological criteria, in order to evaluate the likelihood of

the presence of an overlapped rhinitis. Schematically, cytological features of overlapped rhinitis could be the finding of eosinophilia  $> 20\%$  and/or mast cells  $> 10\%$  in the rhinocytogram of patients with perennial CR and of patients with seasonal CR outside the pollen period corresponding to the personal allergic sensitization<sup>[78,86]</sup>.

The possibility to unveil an overlapped rhinitis through nasal cytology might ameliorate the clinical and therapeutic management of these patients and also could have implications in the epidemiological analysis of pediatric chronic rhinitis and their complications (e.g., asthma, nasal polyposis, chronic rhinosinusitis, etc.). Adult studies suggested that the presence of inflammation and the type of inflammatory cells in NAR influences the clinical features and the risk of comorbidities<sup>[52,76]</sup>.

Indeed, AR and NAR could be overlapping condition in a greater number of cases than previously thought. Distinguishing isolated AR and overlapped rhinitis might improve the clinical management, as those recognize different clinical presentation, comorbidities and therapeutic responses. However, in order to achieve a better correlation between cyto-pathological patterns and clinical features of CR, a systematic application of nasal cytology and further studies are needed, both in adults and children. That might lead to a wider understanding of nasal pathophysiology, to a better classification of nasal diseases and, finally, to a rational therapeutic approach.

## THERAPEUTIC MANAGEMENT

Once the diagnosis of AR has been established, the therapeutic approach includes the avoidance of allergen exposure, whether it is practicable, and the control and/or prevention of nasal symptoms by local or systemic anti-histamine drugs, intra-nasal steroids, leukotriene-receptor antagonists and, in a lesser extent, intra-nasal cromones and decongestants. Among those drugs, intra-nasal steroids have been demonstrated to be able to produce the greatest relief, as those mainly improve the nasal obstruction. Unfortunately, all these drugs control the symptoms, but cannot cure the allergic disease<sup>[87]</sup>.

According to the "allergic/atopic march" hypothesis and to the "united airways disease" concept, AR can be associated to lung function test abnormalities and/or anticipate the onset of asthma. Thus, an important aim of the therapy of AR with identifiable allergenic triggers should be also prevention of the progression to asthma or other respiratory diseases, in addition to ameliorating patient's life quality. Such a goal may be reached through the inclusion of specific immunotherapy (SIT) in the early treatment of AR: Indeed, SIT - unlike symptomatic drugs - has been demonstrated to modulate the immune mechanisms underlying the allergic disease and, therefore, it is the only treatment that currently could modify the natural history of allergic diseases<sup>[88]</sup>. SIT has been administered by two main ways: Subcutaneously (SCIT) or sublingually (SLIT). In



many European countries, SCIT is still the most common way to administer allergy immunotherapy; however, sublingual immunotherapy (SLIT) has been gaining success, especially in the pediatric population, where it could be preferred because it is easily accepted and it is basically lacking of systemic and life-threatening adverse reactions<sup>[89,90]</sup>. Moreover, several systematic reviews supported the specific use of SLIT in the treatment of AR in children, as well as the EAACI position paper on pediatric rhinitis did. Recent analyses inferred a moderate strength and general evidence that SLIT improves pediatric AR and conjunctivitis, ameliorating symptoms and/or decreasing the drug consumption<sup>[12,91-93]</sup>. This evidence resulted to be stronger for grass pollen SLIT in the treatment of isolated AR, whereas the evidence for house dust mite SLIT effectiveness is still considered "of moderate-low quality", as resulted from few available randomized controlled trials. Anyway, some indirect beneficial effects, as the prevention of asthma development and the reduction of respiratory infections, must be considered<sup>[94,95]</sup>. Moreover, as discussed previously, it must be reminded that the importance of a correct diagnostic definition of pediatric chronic rhinitis might affect these conclusions on the efficacy of SLIT. By instance, NAR sustained by the exposure to environmental pollutants producing similar nasal inflammatory changes and disease as house dust mite AR might could affect the outcome analysis of SLIT, by worsening the nasal inflammation due to allergy or by misleading the correct diagnosis. So far, very few studies faced this topic, but an interesting study showed that the exposure to passive smoke significantly reduced the clinical response to SLIT in children affected with AR due to house dust mite<sup>[96]</sup>.

On the contrary, achieving a diagnosis of LAR in patients affected with NAR could have a major impact on the therapeutic management. Indeed, the cornerstone therapy of AR produced a good clinical response in patients with LAR and, particularly, those could receive further important benefit from SIT too. Rondón *et al.*<sup>[97]</sup> reported a significant improvement of symptom and medication scores in 20 patients affected with LAR sensitized to grass pollen after receiving SCIT. However, so far there no pediatric studies regarding drug therapy and SIT in LAR.

As regards the treatment of NAR, the list of drugs basically includes most of molecules used in the management of AR. Unfortunately, patients with NAR resulted to be less responsive to the pharmacological therapy than patients affected with AR, in general<sup>[98]</sup>.

Antihistamines have been largely used for the treatment of NAR. Compared to AR, non-sedating second generation molecules have not resulted to be so effective, whereas some benefits have been observed with first generation antihistamines, probably due to the greater anticholinergic activity. Actually, several studies supported the effectiveness of topical antihistamines in NAR, especially azelastine. Nasal spray containing azelastine have been approved for use in children aged

six years and older, but there are no pediatric studies assessing its efficacy in children with NAR<sup>[98-100]</sup>. The association of azelastine with an intranasal corticosteroid, usually fluticasone propionate, resulted even more effective in the treatment of adult NAR. Similarly, that association was approved for use in children older than five years; however, the available studies on this drug included children aged 12 years and older, which suggested a similar efficacy in AR and NAR<sup>[101,102]</sup>.

Thus, antihistamines and/or topical steroids are the mainstay of the general treatment of NAR. Additional drugs have been used with specific indications. By instance, topical anticholinergic medications, containing ipratropium bromide, could be recommended for patients having rhinorrhea as isolate or dominant nasal symptom, namely vasomotor rhinitis. Specific studies addressing the efficacy of ipratropium nasal spray in pediatric NAR are lacking; however, it has been studied in children complaining of rhinorrhea because of allergy or common cold and resulted to be easy to be administered, safe and effective also in children as young as 2 years<sup>[103,104]</sup>. In children saline nasal irrigation is considered the first step and the basic tool in every therapy for rhinitis and, indeed, resulted to be useful in the management of NAR, too. This procedure resulted to improve nasal symptoms, relieving post-nasal drip, nasal congestion and also sneezing. Nasal irrigation with isotonic saline irrigation seemed to ameliorate the mucociliary clearance, promoting the removal of allergens, biofilms and inflammatory mediators<sup>[6,105]</sup>.

Finally, several adjunctive therapies have been proposed in adults, such as topical capsaicin, anti-leukotrienes and oral/intranasal decongestants. Unfortunately, there is no experience in the pediatric field at all<sup>[99,106]</sup>. However, some recommendations (based upon personal observations) have been reported in Table 2, according to the inflammatory pattern recovered by the nasal cytology.

## CONCLUSION

Chronic rhinitis is a very common medical issue in children, as its incidence was estimated to be 40% in Western countries. Allergic rhinitis is considered to be the most frequent form of chronic rhinitis in children, whereas non-allergic rhinitis resulted to account for at least 25% of cases, according to few available pediatric studies. Actually, pediatric non-allergic rhinitis is poorly defined and is likely to be underrated. Indeed, although local allergic rhinitis is often misdiagnosed as being non allergic, a lot of chronic rhinitis do not receive the appropriate attention after an allergic cause has been excluded. Moreover, cases of non-allergic rhinitis could be lost because of a misdiagnosis of allergic rhinitis or because of its coexistence with allergic rhinitis, defined as mixed or overlapped rhinitis. Currently, the diagnostic definition of pediatric non-allergic rhinitis is often limited to the exclusion of allergy, as several investigations, such as nasal provocation tests and objective measurement of nasal patency, are not suitable for routine

**Table 2** Recommendation for the treatment of pediatric non-allergic rhinitis, according to nasal cytology

	NARES	NARESMA	NARMA	NARNE
Topical anti-histamines	1	1	1	2
Systemic anti-histamines	1	1	1	2
Topical corticosteroids	1	1	1	1
Systemic corticosteroids	1	1	1	2
Ipratropium bromide	2	2	2	2
Anti-leukotrienes	1	1	1	2
Cromones	3	3	3	2
Nasal decongestant	3, 4	3, 4	3, 4	3, 4
Nasal saline irrigation	1	1	1	1

1: Recommended; 2: Not recommended; 3: Uncertain recommendation; 4: Permitted in children younger than 12 years, according to the dosage and for no more than 10-14 d. NARES: Non-allergic rhinitis with eosinophilia syndrome; NARMA: NAR with mast cells; NARNE: NAR with neutrophils; NARESMA: NAR with eosinophils and mast cells.

clinical practice with children. Actually, important clues could come through the growing application of nasal cytology in the diagnostic approach of children complaining of chronic rhinitis. That might lead to a better definition and classification of pediatric non-allergic rhinitis and, as a consequence, to an appropriate clinical management.

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Clinical Trials Study

## Radiological clinical trials: Proposal of a problem-finding questionnaire to improve study success

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### Abstract

#### AIM

To develop a survey to help define the main problems in radiological clinical trials.

#### METHODS

Since 2006, we have managed seven different radiological clinical trials recruiting patients in academic and non-academic centres. We developed a preliminary questionnaire using a four-round Delphi approach to identify problems occurring in radiological clinical trials run at our centre. We investigated the recruitment experience, involvement of all multi-disciplinary team members and main obstacles to completing the projects. A final round of Delphi processes elucidated solutions to the identified problems.

## RESULTS

Among 19/20 (95%) respondents, 10 (53%) were young physicians (under 35 years old), and the respondents included non-faculty members, fellows, residents, and undergraduate students. Ninety-four percent (18/19) of respondents showed interest in conducting clinical trials. On a scale of 1 to 10, the problems with higher/worse scores (8-9) were related to technical or communication problems. The most frequent problems across all studies were technical problems related to clinical trial equipment, insufficient willingness to participate, obstacles to understanding the design of electronic-case report form and extra work.

## CONCLUSION

The developed questionnaire identified the main recurring problems in radiological clinical trials as perceived by end-users and helped define possible solutions that are mostly related to having dedicated clinical trial research staff.

**Key words:** Clinical trials; Data management; Magnetic resonance imaging; Mammography; Ultrasonography

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**Core tip:** Clinical data management (CDM) is important for efficiently managing and completing a clinical trial. CDM is the process of controlling, processing, validating and querying data generated in a clinical study. In this paper, we developed a questionnaire identifying the main recurring obstacles in radiological clinical trials as perceived by end-users. We tried to define possible solutions that are mostly related to having dedicated clinical trial research staff. This topic is relatively well-known by clinicians, while it is less well-known by radiologists and could be useful for radiological centres that are currently involved or will be involved in conducting or participating in radiological clinical trials. For this reason, we suggested a problem-solving questionnaire and reported our experience in managing seven multi-centre national and international radiological clinical trials.

Valdora F, Bignotti B, Calabrese M, Houssami N, Tagliafico A. Radiological clinical trials: Proposal of a problem-finding questionnaire to improve study success. *World J Methodol* 2016; 6(4): 214-219 Available from: URL: <http://www.wjgnet.com/2222-0682/full/v6/i4/214.htm> DOI: <http://dx.doi.org/10.5662/wjm.v6.i4.214>

## INTRODUCTION

Clinical data management (CDM) is important for efficiently managing and completing a clinical trial. CDM is the process of controlling, processing, validating and querying data generated in a clinical study. Recommendations indicate that a specialized research unit may be useful for conducting clinical trials<sup>[1-3]</sup>. As indicated by Farrell *et al*<sup>[3]</sup>, the success

of a clinical trial depends on the presence of an efficient trial team consisting of various experts with different roles and responsibilities. In addition, it is important to have the resources to manage the study workflow stages, as defined by the coordinating centre. The presence of a dedicated trial manager is also important to collecting high-quality clinical data in healthcare studies. Indeed, the collection of poor quality data or the collection of a lower level of data than expected may contribute to underpowered, inconclusive or misleading results.

A good study design and efficient CDM Plan (CDMP) are important for taking full advantage of research project budgets, especially in multi-centre and international collaborative trials. The essential components of a CDMP include the following: Details of study personnel involved in the study and data access roles assigned to each, database design and database location, data entry procedure, methods of data collection - paper or electronic-case report form (e-CRF), data preparation before entry into the electronic system, and data flow and tracking to ensure optimal data completion and facilitate reporting.

The efficiency of the CDMP is crucial to optimizing patient recruitment and follow-up, increasing the percentage of completed e-CRFs, and using processes ensuring that high-quality data are collected with minimal or no missing data. As recently reported<sup>[4-6]</sup>, investigators conducting randomized controlled trials (RCTs) use different strategies to avoid biases in data collection. However, many trials do not recruit sufficient participants, limiting the use of research results and translation of research findings into practice<sup>[7-9]</sup>. Additionally, an audit is necessary to regularly monitor the randomization process<sup>[10,11]</sup>. Standardized procedures are necessary to handle errors or problems in the randomization process and data acquisition, which is crucial to the overall trial quality.

The medical literature lacks a structured description of the main problems affecting clinical trials that specifically deal with imaging and are led by a radiological unit<sup>[12]</sup>. Imaging in research is increasingly involved. The use of imaging data in clinical research can provide many scientific benefits, but it can result in additional complexities that contribute to risks, biases and errors<sup>[13]</sup>. As indicated by Erickson *et al*<sup>[14]</sup>, the use of imaging data in clinical trials may be a part of the solution for reducing the cost and increasing the efficiency to conduct a timely clinical trial. A frequent problem with a radiological clinical trial consists of the quality of the clinical trial data; multi-centre clinical trials need reproducible, quality assured data with post-processing methods supported by an operational infrastructure.

In the hospital, the medical subject's imaging data are managed in the clinical picture archiving and communication system (PACS) *via* the digital imaging and communications in medicine (DICOM) protocol. Clinical PACS could be separate from the research PACS.

PACS is extremely limited in its support for research imaging. The system is DICO-centric and generally does

not support the alternative file formats used in research. It is essential to guarantee the high quality of the entire process that images for clinical trials are collected using uniform image acquisition and measurement methods to minimize the variability.

To address this knowledge gap, we performed a survey-based study to identify the main problems in conducting radiological clinical trials and to help find solutions, including roles for staff dedicated to ongoing radiological clinical trials. The aim of this study was to identify potential barriers to conducting clinical trials in imaging.

This work is a pilot study. The survey was performed as an internal questionnaire survey at our centre, which is involved in several multi-centre clinical trials, and the preliminary results could help all centres involved in radiological clinical trials find solutions to the main problems and improve the progress and outcomes of future radiological clinical trials.

## MATERIALS AND METHODS

### *Clinical trials*

Data for this study were derived from staff involved in seven different radiological national and international multi-centre clinical trials employing cancer imaging. The clinical trials are listed as indicated in the Supplementary Information. The first study was performed in 2006 and the most recent in 2015<sup>[15-18]</sup>.

All studies included in this work were already approved by the respective Ethical Committee and all participants signed a written informed consent form before enrolment. The studies were performed according to the principles outlined in the Declaration of Helsinki.

The studies codified as ASTOUND<sup>[15]</sup>, Tomo-micro<sup>[16]</sup>, BP-US<sup>[17]</sup> and BP-MRI<sup>[18]</sup> in the Supplementary Information were already published.

### *Development of the survey*

The survey was developed using a 4-step consensus approach by the Delphi method<sup>[19,20]</sup>. The personnel of the University Hospital and all teams that participated in the seven radiological trials were invited to respond to the survey and participate in the Delphi method. The Delphi method is based on the premise that collective beliefs are more trustworthy than the beliefs of a single person; therefore, it is considered an efficient procedure to generate thematic knowledge<sup>[20]</sup>. By this method, opinions, expertise and critical thinking are systematized. Individual feedback on a topic, the judgment of the group's work, and opportunities to change opinion were given in an anonymous form<sup>[19]</sup>. The questionnaire focused on the key issues identified by the personnel directly involved in the trials to reduce the influence of department chairs.

The first step consisted of a review of the existing literature up to July 2015 and the development of the first draft of the survey. The subsequent three steps each included a Delphi round to develop the final survey. A

series of discussions (face-to-face meeting and e-mails) among the participants was performed. The survey investigated several stages of clinical trials, including the recruitment experience, effective involvement of all multi-disciplinary teams (MDTs), the main obstacles faced in clinical trials, and the background of each team member. After the survey, critical issues were identified and summarized; then, possible solutions were suggested by the same Delphi method.

The questionnaire consisted of 12 items that were written in English, as indicated in Supplementary Figure 1.

We have classified each issue of the survey given to the participants with a score of 0 to 10 (1 = no problems observed, 10 = several problems can negatively affect the results and induce the participants to quit). The characteristics are listed in Table 1.

### *Survey participants*

The survey involved investigators who were participants belonging to the MDT, including personnel of the University Hospital and of all teams who took part in the seven radiological trials as described above. They were asked to complete the questionnaire, highlighting the main problems faced during clinical trials.

The survey was sent to all clinical team members, including the principal investigators (PIs), research nurses, nursing staff, and technicians. The anonymous questionnaire had to be returned to the identified PI's delegate to record the responses, as normally done in a Delphi process. We performed further rounds of Delphi processes to solve all encountered difficulties.

### *Statistical analysis*

The mean experience of team members in radiological clinical trials as well as the percentage of questionnaires returned was recorded. Group agreement with the clinical condition under consideration was defined as total cumulative agreement > 67% after the second or third Delphi round. Group consensus was defined if the consensus level of agreement (CLA) was > 90% for each issue of the survey. The results are presented as the total cumulative agreement after the last Delphi round by a four-point simplified Likert scale (agree, agree with minor reservation, agree with major reservation, and disagree).

## RESULTS

### *Characteristics of survey participants*

Nineteen of 20 team members (95%) returned the questionnaires. Ten of nineteen of survey participants were young physicians and non-faculty members (fellows, resident and undergraduate students). The other members (9/19) were staff-doctors, principal investigators, and co-investigators. Additionally, 18/19 of respondents showed interest in conducting clinical trials. Among these, a large proportion of physicians with previous clinical trial experience (14/18) and many residents, data managers, and nurses without clinical trial experience expressed high interest in conducting clinical trials. Only one participant



**Table 1** List of main issues and problems identified when conducting clinical trials<sup>1</sup>

Problem	Score (mean $\pm$ SD) among respondents	Effect on clinical trial conduction	Suggested solution	No. of surveys scored from 19 completed surveys
Principal investigator	9 $\pm$ 0.5	Lack of team consistency and participation	The principal investigator should be PERSONALLY involved and have a pro-active approach to the study	15/19
Administrative impediments (ethics committee, insurance) affect the beginning of clinical trials	6 $\pm$ 0.37	Delay in starting the study	Employ a coordinator from administrative staff with no clinical burden	13/19
Technical problems with instruments used in the study	6 $\pm$ 0.62	Delay in conducting the study	Identify a key person to regularly check instrumentation	12/19
Insufficient willingness to be part of a team and to collaborate in the trial	7 $\pm$ 0.41	Lack of interest and enthusiasm and inability to progress or finish in time	Organise frequent investigator meetings, conference calls and study checks	15/19
Slightly different clinical practices of the involved centres	7 $\pm$ 0.42	Risk of missing or non-standardized data	Discuss and standardize practical, methodological data-related aspects of the study	14/19
Difficulties to complete a complex e-CRF	7 $\pm$ 0.46	Incomplete e-CRF and missing data	Simplify the e-CRF	17/19
Perform quantitative evaluations	8 $\pm$ 0.38	Delay in quantitative radiological data acquisition	Have dedicated trained personnel and workstations	18/19
Extra work required to comply with study inclusion criteria	9 $\pm$ 0.32	Loss of patients potentially eligible for the study	Check inclusion criteria in advance by available patient data review	18/19

<sup>1</sup>The score system ranges from 1 (no problem) to 10 (serious problem). e-CRF: Electronic-case report form.

was involved in a clinical trial that had terminated before the completion of the present survey.

### Main problems encountered

The main barriers faced in conducting a radiological clinical trial (with a score of 8-9) were the time commitment to perform quantitative evaluations of radiological exams that are already reported and the extra work required to comply with the clinical trial's inclusion criteria. A score of 6, reflecting a significant but not severe problem, was the need to deal with administrative impediments, such as the need to prepare all the documents for the local ethics committee and insurances for research studies. Indeed, these problems can delay the beginning of the radiological clinical trial. A low score of 6 was also due to a technical problem with the instruments (for example, new software applications) needed in a study and the lack of organized support from the hospital facilities. A score of 7 indicated a possible lack of interest to conduct the clinical trial and several difficulties to complete the e-CRF. From participating in multinational clinical trials, 15/19 of respondents assigned a score of 8 or 9 for the PI, indicating that the role of the PI is crucial to conducting a radiological clinical trial. After problem identification, possible solutions suggested from the final Delphi round are reported in Table 1.

## DISCUSSION

Clinical trials have rapidly evolved during the past decade. As we discussed above, radiological clinical trials can be

affected by different types of bias concerning imaging technology and recruitment strategies. Bias can result from differences in the methods in which information is collected or in the manner in which data are obtained during the recruitment process. In the past, radiologists have had limited direct patient interaction and have depended on other specialists to refer patients for enrolment; in this way, inadequate approaches to patient recruitment could introduce bias. The main strategies for recruitment were flyer distribution, brochure pick-up, internet posting-ads or poster distributions without direct patient contact. Current technology has allowed us to take a different approach, directly interact with the patients, and monitor the follow-up or response.

In this work, we developed a preliminary survey to elucidate knowledge on obstacles or problems in running radiological clinical trials from all participating in various radiological studies at our centre, and we hoped that the acquired information could improve the conduct of radiological clinical trials. We observed that several obstacles (related to administrative, technical/equipment, or resourcing issues) could hamper the development of relatively feasible radiological clinical trials. Using the same survey-based/Delphi process, we also sought to define possible solutions to the main problems that had to be overcome during several radiological clinical trials.

We tried to differentiate serious problems from less serious or minor problems. It is not surprising that the majority of problems that received a high score were related to the lack of resourcing and, specifically, to the lack of dedicated research personnel without a clinical

burden. Indeed, busy daily radiological clinical practices have limited time for the additional work generated by conducting or contributing to a clinical trial. In our survey, the highest scores ("bigger problems") were assigned to issues that typically go well beyond the radiological report, such as performing a quantitative evaluation on radiological images as part of the research protocol, or becoming familiar, and complying with the study inclusion criteria (patient eligibility). Indeed, for prospective trials, respecting the inclusion criteria of the study is crucial for several reasons, such as reaching the required number of patients and collecting reliable and unbiased data. Consequently, the suggestion given by the last Delphi round was to have dedicated clinical trial personnel who are not involved in the clinical routine undertake the role of checking and ensuring compliance with the inclusion criteria.

In larger multicentre studies requiring that data are gathered from different centres, even minor differences in the population, culture, nomenclature and medical practice can be causes of variability. As indicated by Willis-Shattuck *et al.*<sup>[21]</sup> in a systematic review facing the issues related to recruitment in developing countries, the authors reviewed all studies investigating the link between motivation and retention of health workers in developing countries. The authors concluded that motivational factors are influenced by the context, and the successful completion of a study depends on the number of available resources.

It is commonly thought that the public health sectors of many countries suffer from a surplus of workers who are not particularly productive because they have not received adequate training. In fact, a survey presented at RSNA 2013 by Rehani *et al.*<sup>[22]</sup> confirms that radiologists in developing countries need an accurate training program.

Indeed, in a single country, multi-centre trial, there can be variability and bias, but some of the possible sources of bias can be controlled with an appropriate trial design. An important result of our survey is that standardized trial planning and the identification of a key figure managing several phases of a radiological clinical trial is very important for ensuring a timely start and correct development of the trial.

Through our experience of being involved in seven different studies on cancer imaging and collaborating with several research groups from different contexts, we investigated how many problems can arise when developing clinical studies. Unfortunately, we did not evaluate the hospital due to a lack of funding.

We found it very useful to monitor monthly enrolment progress by site and permit sites to compare and discuss their progress. We organized collaborative workshops with all investigators from the included studies for all periods of the studies. These meetings were valuable to discuss practical, methodological and data-related aspects of each original study and to build trust among investigators. During these workshops, we discussed and refined the study protocol in advance, examined patient

characteristics and information from diagnostic tests that are to be analysed, and agreed on data checking procedures and the main analyses to be performed.

In conclusion, this study could be a valuable preliminary survey that can elucidate the critical key points identified in radiological clinical trials. Obviously, this study does not solve all problems that a radiologist could face during a clinical trial. However, the main problems in oncology clinical trials or in imaging are not very different, and they are in common with what has previously been described as essential to successfully concluding a clinical trial. It is important to identify the crucial role of key people who are capable of connecting different expertise levels and responsibilities. Indeed, each person involved in conducting a trial should be instructed and qualified to tailor his or her respective task(s), taking advantage of previous cultural backgrounds. Our problem-solving approach may improve the organization of radiological clinical trials, especially in non-academic centres.

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## COMMENTS

### Background

Clinical data management (CDM) is the process of controlling, processing, validating and querying data generated in a clinical study. Recommendations indicate that a specialized research unit may be useful for conducting clinical trials. The presence of a dedicated trial manager is important for collecting high-quality clinical data in healthcare studies. Indeed, the collection of poor quality data or of a lower level of data than expected may result in underpowered, inconclusive or misleading results. The essential components of a CDM plan (CDMP) include: Details of study personnel involved in the study and data access roles assigned to each, database design and database location, data entry procedures, methods of data collection - paper or e-CRF, data preparation before entry onto electronic system, and data flow and tracking to ensure optimal data completion and facilitate reporting.

### Research frontiers

The efficiency of the CDMP is crucial to optimizing patient recruitment and follow-up, increasing the percentage of completed electronic-case report forms, and using processes ensuring that high-quality data are collected with minimal or no missing data. Usually, investigators conducting randomized controlled trials employ different strategies to avoid biases in data collection. Standardized procedures are necessary to handle errors or problems in the randomization process and data acquisition, which is crucial to the overall quality of the trial.

### Innovations and breakthroughs

A good study design and an efficient CDMP are important for taking full advantage of research project budgets, especially in multi-centre and international collaborative trials. The information from this study might allow all centres involved in radiological clinical trials to find solutions to the main problems as well as help improve the progress and outcomes of future radiological clinical trials.

### Applications

The medical literature lacks a structured description of the main problems that affect clinical trials specifically dealing with imaging in a radiological unit. The aim of this study was to identify potential barriers to conducting clinical trials in imaging.

## Peer-review

The authors investigated problems faced when conducting clinical trials. This work clarified the issues for improving the efficiency of clinical research.

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