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Contents

Quarterly Volume 5 Number 3 August 20, 2015

**Contents**

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EDITORIAL

# Interferon-γ: Promising therapeutic target in atherosclerosis

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## Abstract

Atherosclerosis is a chronic inflammatory disorder of the vasculature and is the primary cause of cardiovascular disease (CVD). CVD is currently the world's leading cause of death and the numbers are predicted to rise further because of a global increase in risk factors such as diabetes and obesity. Current therapies such as statins have had a major impact in reducing mortality from CVD. However, there is a marked residual CVD risk in patients on statin therapy. It is therefore important to understand the molecular basis of this disease in detail and to develop alternative novel therapeutics. Interferon-γ (IFN-γ) is a pro-inflammatory cytokine that is often regarded as a master regulator of atherosclerosis development. IFN-γ is able to influence several key steps during atherosclerosis development, including pro-inflammatory gene expression, the recruitment of monocytes from the blood to the activated arterial endothelium and plaque stability. This central role of IFN-γ makes it a promising therapeutic target. The purpose of this editorial is to describe the key role IFN-γ plays during atherosclerosis development, as well as discuss potential strategies to target it therapeutically.

**Key words:** Atherosclerosis; Interferon-γ; Inflammation; Neutralization; MicroRNA



**Core tip:** Atherosclerosis is an inflammatory disorder of the vasculature and studies in mouse model systems have highlighted the beneficial effects of counteracting inflammation in limiting the progression of this disease. Due to its key role in inflammation and atherosclerosis development, interferon-γ (IFN-γ) is seen as a promising therapeutic target. In this editorial we discuss the role of IFN-γ in atherosclerosis together with potential therapeutic approaches against this cytokine and its key downstream targets.

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## INTRODUCTION

Atherosclerosis is the underlying cause of cardiovascular disease (CVD) such as myocardial infarction (MI) and

stroke. The World Health Organisation estimated that there were 17.5 million deaths from a CVD-related event in 2012, equating to approximately 1 in 3 global deaths[1]. The number of global deaths related to CVD has been predicted to increase due to rises in the incidences of obesity and diabetes and the acquisition of a westernised diet in developing countries. The disease is a major healthcare and economic burden and therefore there is a need to understand the disease in more detail and to develop new therapeutic approaches.

## ATHEROSCLEROSIS DEVELOPMENT

Atherosclerosis is a chronic, inflammatory disease characterized by the formation of foam cells in initial atherosclerotic lesions which then progress into advanced plaques. Low-density lipoprotein (LDL) can become trapped in the intima of medium and large arteries and modified to oxidized LDL (OxLDL). The presence of OxLDL triggers an inflammatory response in the neighbouring endothelial cells (ECs), causing the release of a variety of pro-inflammatory cytokines and chemokines, and expression of adhesion molecules on the cell surface (activation of ECs). These factors include macrophage chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) as well as P- and E-selectins[2,3]. Such pro-inflammatory molecules guide circulating monocytes in the blood stream to the OxLDL accumulation in the intima of arterial walls and aid the progression of atherosclerosis development[4-6]. Once in the intima the monocytes become exposed to macrophage colony-stimulating factor, triggering their differentiation into macrophages as well as inducing scavenger receptor (SR) expression on their surface[2,7]. Macrophages are then able to uptake OxLDL by SR-mediated endocytosis, macropinocytosis or phagocytosis and develop into foam cells, causing the appearance of the initial lesions and fatty streaks in arteries, which can then progress into mature plaques[8,9].

Mature atherosclerotic plaques are made up of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM), as well as accumulated OxLDL, cholesterol and apoptotic cells, which form a lipid-rich necrotic core[10]. During plaque progression VSMCs proliferate and migrate towards the LDL accumulation and form a fibrous cap, which is tightly controlled and influenced by the nearby macrophages, ECs and T-cells[2,11]. As the fibrous cap continues to develop it forms a stable lesion by covering the large lipid-rich necrotic core, therefore the balance of ECM production and degradation can affect the stability of the lesion[2]. If the plaque ruptures it triggers a thrombotic reaction and in turn platelet aggregation, which can quickly impede or obstruct blood flow through the artery[7]. Depending on the location of the rupture it can potentially cause a MI or stroke. Therefore acute CVD events may be manageable by affecting plaque stability and preventing them from rupturing[7,12]. Amongst the cytokines involved in the development of atherosclerosis, interferon-γ (IFN-γ) is potentially a master regulator and will therefore be addressed in more detail.

## IFN-γ

IFN-γ is a key pro-inflammatory cytokine in atherosclerosis development as it is capable of inducing the expression of approximately a quarter of genes expressed in macrophages[3]. Immune cells present in the atherosclerotic lesions, including T-lymphocytes, natural killer T-cells, macrophages and other antigen presenting cells, secrete IFN-γ at pronounced levels[13,14]. Stimulation of many signaling pathways that regulate the immune and inflammatory responses can be induced by IFN-γ. The major signaling pathway that IFN-γ signals through is the Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) pathway[3].

### *JAK-STAT pathway*

The IFN-γ cell surface receptor complex (IFN-γR) is made up of two subunit pairs (IFN-γR1:IFN-γR) which dimerize upon binding of the cytokine[13]. Bound to each subunit are two JAKs 1 and 2, which become activated by phosphorylation of tyrosine residues in the N-terminus in a mainly JAK2-dependent process[15]. Once activated, the JAKs phosphorylate the tails of the IFN-γR which triggers the recruitment of STAT1 monomers from the cytoplasm that then interact with the receptor *via* their src-homology 2 domains[16]. The recruited STAT1 monomers are then phosphorylated by the JAKs at tyrosine 701 and dissociate from the receptor complex to form STAT1:STAT1 homodimers[3]. The dimer is then able to translocate into the nucleus and stimulate the transcription of IFN-γ target genes, such as MCP-1 and ICAM-1, by binding to γ-activated sequence (GAS) elements in their promoters[13,15]. Furthermore, extracellular signal-regulated kinase (ERK) and other kinases are capable of phosphorylating the homodimer at serine 727 for maximal activity[17].

## ROLE OF IFN-γ IN ATHEROSCLEROSIS DEVELOPMENT

Therapeutically targeting IFN-γ in order to reduce the incidence of CVD represents a promising avenue due to its pro-inflammatory functions during atherosclerotic plaque formation, including the recruitment of immune cells to the site of OxLDL accumulation, foam cell formation, and plaque development and stability. A 2-fold increase in the size of atherosclerotic lesions has been reported in the Apolipoprotein E (ApoE) deficient mouse model that was injected with recombinant IFN-γ every day, even with a 15% reduction in plasma cholesterol levels[18]. Furthermore, ApoE deficient mice which also lacked IFN-γR showed a reduction in atherosclerosis development, as well as a 60% decrease in lipid

build up in the lesions when fed on a western diet[19]. Deficiency of STAT1 in mouse model systems is also associated with reduced atherosclerosis development and foam cell formation, highlighting the key role of the JAK-STAT1 pathway in IFN-γ signaling during plaque progression[20,21].

### *Recruitment of immune cells*

IFN-γ is a key recruiter of immune cells in the development of atherosclerosis and therefore important in the growth of lesions[22]. IFN-γ has been shown to be localized in atherosclerotic lesions and mice models lacking either IFN-γ or its receptor have been reported to have a reduced cellular content in their lesions[19,23,24]. The expression of key pro-atherogenic chemokines and their receptors, such as MCP-1 that has been detected in atherosclerotic lesions by immunohistochemistry and *in situ* hybridization, can be induced by IFN-γ[25,26]. Mouse models which were deficient for either MCP-1 or its receptor showed a reduced cellular content in lesions, as well as a reduction in the size of the lesions without changes in circulating lipid or lipoprotein levels[25]. IFN-γ can also influence the recruitment of immune cells by inducing the expression of adhesion molecules, such as ICAM-1 and VCAM-1, in ECs during the early stages of atherosclerosis development[27,28].

### *Foam cell formation*

Cholesterol uptake and efflux is carefully balanced during homeostasis of this sterol in healthy cells. The formation of foam cells can be regarded as a pathological imbalance in favour of reduced cholesterol efflux and increased uptake of OxLDL[7,29]. The expression levels of a number of key genes involved in cholesterol metabolism are regulated by IFN-γ, including ApoE, ATP-binding cassette transporter A1 (ABCA1) and acetyl-CoA acetyltransferase 1 (ACAT1)[22]. *In vitro* studies that have incubated macrophage-derived foam cells with IFN-γ have shown a reduction in cholesterol efflux *via* increasing the expression of ACAT1 and attenuating the expression of ABCA1, resulting in increased accumulation of intracellular cholesteryl esters which promote the formation of foam cells[30]. Furthermore, the expression of several key SRs in foam cell development, including SR-A and SR that binds phosphatidylserine and oxidized lipids (SR-SPOX; also known as CXCL16), have been shown to be increased in human THP-1 and primary macrophages stimulated with IFN-γ, resulting in an increased uptake of OxLDL[31-33]. Therefore IFN-γ is capable of altering cholesterol homeostasis towards lower cholesterol efflux and higher retention of OxLDL in macrophages and contributes to foam cell formation.

### *Plaque progression and stability*

IFN-γ can influence a variety of processes involved in the development of the early atherosclerotic lesions into mature plaques as well as their stability. Part of plaque development involves the migration of VSMCs and the formation of the fibrous cap. IFN-γ induces the expression of integrins on the surface of VSMCs which are capable of binding to fibronectin in ECM, triggering the VSMCs to differentiate from their inactive to their proliferative phenotype allowing migration towards the lesion to form the fibrous cap[34]. The stability of atherosclerotic plaques relies on the balance of ECM production and degradation which can also be affected by IFN-γ[2,22]. Foam cell apoptosis is also promoted by IFN-γ and causes them to expel their contents into the intima, contributing to the lipid-rich necrotic core and ECM degradation[35,36]. The balance can be tipped further towards ECM degradation by IFN-γ-mediated inhibition of the expression of several collagen genes, thereby suppressing matrix synthesis by VSMCs and resulting in reduced plaque stability and increased risk of a rupture[7]. ECM degradation can also be triggered by matrix metalloproteinases (MMPs) which are found in atherosclerotic plaques and are often localized to the shoulder regions where a rupture is more likely to occur[37]. MMPs are released by macrophages and VSMCs and their expression can be induced by IFN-γ stimulation[38].

## THERAPEUTICALLY TARGETING IFN-γ

Due to the high prevalence of CVD there are a variety of therapeutics designed to reduce various aspects of atherosclerosis development, including decreasing serum cholesterol levels and altering the expression of genes that are involved in cholesterol metabolism or the inflammatory response[3,39]. Statins, the most widely used and successful cholesterol lowering therapy class of drugs, are primarily designed to inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG CoA reductase)[3]. HMG CoA reductase catalyses the rate limiting step in cholesterol biosynthesis, thereby lowering the levels of circulating LDL[40]. However there is a marked residual risk of CVD in patients on statin therapy, with a significant proportion unable to attain their target LDL levels even when receiving the highest recommended dosage, stressing the importance of developing new therapeutics[2,41].

One new potential therapeutic target is IFN-γ due to its key roles in atherosclerosis development. There are currently two strategies that have been developed that either target IFN-γ directly (IFN-γ neutralization) or inhibit its signaling pathways. Statins and agonists of nuclear receptors also attenuate IFN-γ actions in part by modulating its signal transduction pathways[42-44]. In human macrophages, IFN-γ-induced phosphorylation of STAT1 on serine 727 can be blocked using adenosine[45]. Work by Lee *et al*[46] has shown that stimulation of the adenosine A3 receptor with a novel agonist, thio-CL-IB-MECA, resulted in attenuated IFN-γ-induced STAT1-dependent gene expression. Furthermore a naturally occurring phenol in plant extract, resveratrol, is capable of preventing STAT1 phosphorylation at tyrosine 701

or serine 727 as well as JAK2 activation in human macrophages *in vitro*[47]. These compounds represent promising avenues for therapies targeted at the downstream signaling events in the JAK-STAT pathway in order to reduce the pro-inflammatory effects of IFN-γ. Other therapies target IFN-γ *via* alternative signaling pathways, for example, ACS14 (a hydrogen sulphide releasing aspirin) is capable of attenuating the expression of IFN-γ-stimulated CX3 chemokine receptor 1 (CX3CR1) *via* a peroxisome proliferator-activated receptor-γ-dependent mechanism[48]. Hydrogen sulphide has previously been shown to exert anti-atherogenic effects and its use in ACS14 has been shown to reduce atherosclerosis development in ApoE mice models[48,49].

IFN-γ neutralization involves the use of a soluble IFN-γR (sIFN-γR) which acts as decoy receptor to prevent the activation of IFN-γR and in turn the phosphorylation of STAT1 in the JAK-STAT pathway, in effect "neutralizing" the IFN-γ. The approach was first developed by Koga *et al*[50], and demonstrated in ApoE mice which were fed a high fat diet for 8 wk and given two intramuscular injections of a plasmid encoding sIFN-γR at weeks 4 and 6. Compared to the control mice, those that received the sIFN-γR injections had dramatically reduced atherosclerotic lesion size as well as greater plaque stability. This increase in plaque stability was found to be due to an increase in the number of VSMCs in the fibrous cap in addition to greater collagen deposition. Additionally, there was also a decrease in the amount of lipid accumulation and number of macrophages in the necrotic core, which further improved plaque stability and reduced the risk of rupture. Furthermore, neutralizing antibodies have been used for other cytokines such as IL-1β and show great therapeutic promise[51,52], therefore similar strategies could potentially be developed to use antibodies to achieve IFN-γ neutralization.

Although targeting IFN-γ in atherosclerosis development may result in reduced lesion size and improved plaque stability, there are potential drawbacks that need to be assessed before IFN-γ targeting can be recommended therapeutically. The major concern involves the systemic inhibition of IFN-γ due to the major role it performs in the immune response[53]. Sustained universal inhibition of IFN-γ may increase an individual's risk of acquiring intracellular infections and tumour development[53]. On the other hand it may benefit those high-risk patients who are unable to achieve target LDL plasma levels using currently available therapeutics. A possible solution to overcome universal inhibition would be to try and develop a drug delivery system, for example using nanoparticles, that would allow IFN-γ-targeted therapeutics to be delivered to a specific location rather than system wide[53,54].

Another possible solution would be to target further downstream targets of the IFN-γ signaling pathways, either alone or in combination with therapies that target IFN-γ directly. IFN-γ is known to induce the expression of several microRNAs (miRNAs) in addition to having its own expression regulated by miRNAs[55]. miRNAs are short non-coding single-stranded RNAs approximately 19-25 nucleotides in length that are evolutionary conserved in eukaryotic organisms[56]. Evidence is continuously accumulating that indicates that miRNAs are capable of regulating gene expression by inhibiting translation or inducing targeted mRNA degradation[57]. miRNAs have also been found to regulate a number of key steps during atherosclerosis development, including the inflammatory response triggered by IFN-γ[58-60]. One miRNA that is thought to play a key role in atherosclerosis development is miR-155. Evidence for the role of miR-155 in the inflammatory response was found by O'Connell *et al*[61]. miR-155 was the only miRNA out of 200 tested that was considerably up-regulated in primary murine macrophages after being treated with pro-inflammatory stimulants. Additional evidence for the involvement of miR-155 in the inflammatory response comes from studies which have shown its levels to be up-regulated in macrophages in atherosclerotic lesions as well as having an association with increased pro-inflammatory cytokine expression, potentially due to its ability to repress the expression of the Suppressor of Cytokine signaling 1 (*SOCS1*) gene[62-64]. However the specific role miR-155 plays during atherosclerosis is still being debated, with a number of studies reporting miR-155 to exert pro-atherogenic effects in ApoE deficient mouse models[65,66]. Targeting miRNAs, which are either regulated by IFN-γ and are known to be involved in atherosclerosis development or regulate the expression of IFN-γ, may provide an excellent therapeutic avenue that allows specific arterial targeted treatment to reduce atherosclerosis development and improve plaque stability without potential consequences from systemic IFN-γ inhibition.

## CONCLUSION

Due to the central role of IFN-γ during atherosclerosis development and plaque stability, along with the expected rise in global rates of CVD-related events, this cytokine represents a promising therapeutic target. Targeting either IFN-γ directly or its signaling pathways in both *in vitro* and *in vivo* studies has shown that directed therapies have the potential of reducing atherosclerosis development. However the potential side effects of long term IFN-γ inhibition still needs to be assessed.

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EDITORIAL

# Eurytrematosis: An emerging and neglected disease in South Brazil

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## Abstract

The trematodes of the genus *Eurytrema* are low pathogenic pancreatic parasites, but can be related to a decrease in cattle productive performance and eventually death. Parasitized animals develop chronic interstitial pancreatitis and may show a productive performance drop and emaciation. Human infection by *Eurytrema* sp. has already been reported in other countries as an incidental finding during autopsy or routine tests, but the parasite has not been found in humans in Brazil. However, it is possible that a large number of people could be infected, since parasitological tests have low sensitivity and the parasite is neglected as a pathogen for humans and even animals. Attempts to control and treat Eurytrematosis have generally presented low effectiveness. With the aim to control the disease and provide more information regarding its pathogenicity, our research group is developing a number of studies about *Eurytrema* spp. We hope to determine the damage in productivity, as well as, establish an efficient protocol for treatment and control of Eurytrematosis based on immunoprophylaxis and antiparasitical drug therapy.

**Key words:** Public health; Pathology; Diseases of cattle; Oxidative stress; Parasitical immunoprofilaxis

**Core tip:** The trematodes of the genus *Eurytrema* are low pathogenic pancreatic parasites, but can be related to decrease in productive performance and eventually death of infected animals. Human infection has already been reported but could be underestimated in Brazil. With the aim to control the disease and provide more information regarding its pathogenicity, our research group is developing a number of studies about *Eurytrema* spp. We hope to determine the damage in productivity, as well as, establish an efficient protocol for treatment and control of Eurytrematosis based on immunoprophylaxis and antiparasitical drug therapy.

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## EURYTREMATOSIS: AN EMERGING AND NEGLECTED DISEASE IN SOUTH BRAZIL

The trematodes of the genus *Eurytrema,* belonging to the family Dicrocoeliidae are parasites known as low pathogenic and are generally considered to be an incidental finding at necropsy or at slaughterhouse, but they can be related to a decrease of an infected animals productive performance, and eventually death[1]. Among the species of *Eurytrema* we can highlight *Eurytrema pancreaticum* (*E. pancreaticum*) and *E. coelomaticum,* common parasites of pancreatic ducts of ruminants, occasionally found in the bile ducts and rarely in the small intestine. *E. pancreaticum* has been reported in cattle, goats, sheep, swine, buffaloes, camelids, cervids and human beings, while the *E. coelomaticum* has been reported in cattle, goats, sheep, buffaloes, leporids and camelids[2]. In Brazil the parasites have two different intermediate hosts, the mollusk *Bradybaena similaris* and different species of arthropods from the genus *Conocephalus*[2]. The life cycle is explained in Figure 1.

In areas where the disease occurs, the prevalence among animals is generally high. In a study undertaken by our group[3] we identified a prevalence of 75% of *Eurytrema* sp. in cattle from the West of Santa Catarina State, with 100% of the farms having at least one infected animal. In another Brazilian study, authors found a lower prevalence: between 8.3% and 40.55% in different regions of the State of Paraná (the state north of Santa Catarina) and also observed a seasonal variation in the parasitism[4]. Bassani *et al*[5] found prevalences between 26.9% and 72.9% in the central-west region of Paraná, but in this study a definite seasonal distribution was not observed. Whereas in the State of Rio Grande do Sul (the state south of Santa Catarina) the parasitism by *Eurytrema* sp. is uncommon[6]. In spite of the high prevalence reported in some studies, clinical cases in cattle with emaciation and mortality are sporadic and when occurs affects no more than 3% of the herd[1].

Human infection by *E. pancreaticum* has already been described as an incidental finding at autopsy or in routine coproparasitological tests[7]. There are no published reports in humans in Brazil, but it is possible that a great number of people could be infected, since in general, it is common that routine coproparasitological tests result in false negatives. Similarly to animals, human infection may be subclinical. Another important aspect is that nowadays in medical practice of parasitological diagnosis, many Brazilian physicians request serological tests, instead of the coproparasitological ones, mainly due to the higher sensitivity. However, since *Eurytrema* sp. has not been described as a human pathogen in Brazil, it is not investigated and is likely to be underestimated. Some feeding habits in areas with high prevalence of the parasite seem to be risk factors for human infection. The consumption of wild watercress as salad is common in our region and is an important risk factor for infection by *Fasciola hepatica*[8] and could also be an important risk factor for *Eurytrema* sp.

In Brazil, the pathological pattern of the clinical cases of Eurytrematosis in cattle was described as chronic and progressive weight loss related to interstitial pancreatitis[1,9,10]. In our routine necropsies of cattle that have died for different reasons, in about 92% of the cases *Eurytrema* sp. was found in the pancreatic ducts (Figure 2), most of the times with moderate to severe infections. During histopathology we have observed that the parenchyma of the pancreas is extensively replaced by fibrous connective tissue (Figure 3), which could disrupt the function of the organ.

Attempts to control and treat Eurytrematosis, when applied, generally present low efficiency. This fact seems to be a result of the parasites resistance against the main endoparasiticides used and the high rate of reinfection. Yamamura[11] conducted a trial with four molecules of endoparasiticides commonly used in veterinary practice: nitroxinil, triclabendazole, rafoxanide and closantel, and reported that none of them were efficient. Araújo and Belém[12] reported that albendazole was not efficient against *Eurytrema* sp. in naturally infected cattle, even when administrated in high dosages (17.5 mg/kg bw).

There are few reports about efficient treatments against Eurytrematosis in cattle. Sakamoto *et al*[13] described treatment regimes involving two applications of nitroxinil with an interval of 20 d, and three applications of praziquantel with intervals of two days; both regimes reduced the output of eggs in feces to 0. Jiraungkoorskul *et al*[14] in an *in vitro* trial concluded praziquantel should be the chosen drug for Eurytrematosis control, since it caused the death of 100% of the parasites, while

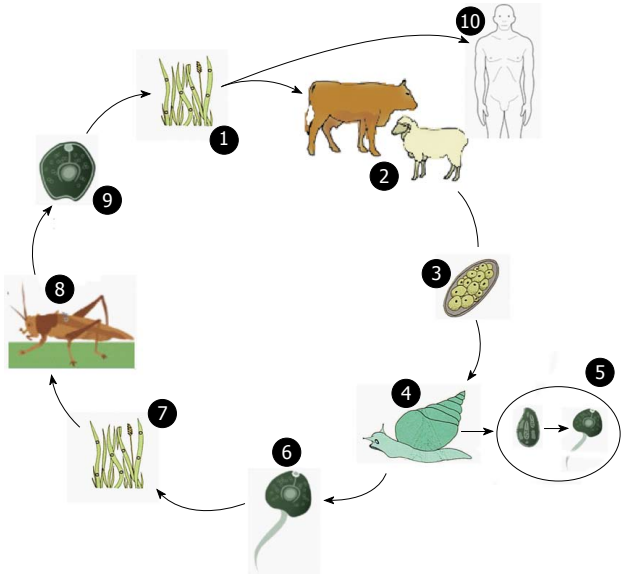


**Figure 1 *Eurytrema* spp. life cycle.** 1: Infection by grazing on infected grass; 2: Vertebrate host (cow or sheep); 3: Unembryonated eggs released in feces; 4: Ingestion of eggs by *Bradybaena* sp.; 5: Sporocyst mother in sporocyst son in the digestive tube between 90 and 350 d, according to envionmental conditions. Sporocyst sons containing cercarea are released in the environment few hours before dawn; 6: Cercarea released in the environment; 7: Ingestion of cercarea by *Conocephalus* sp.; 8: Development of infective metacercarea inside grasshopper; 9: Infective metacercarea released in the environment; 10: Possible human infection by consumption of infected salad. One egg of *Eurytrema* spp. produces 100 sporocyst sons which turn into 200 cercareas and latter 20000 infective metacercarea. Adapted from Tessele, CDC and Bennett[6,15,16]. CDC: Centers for Disease Control and Prevention.

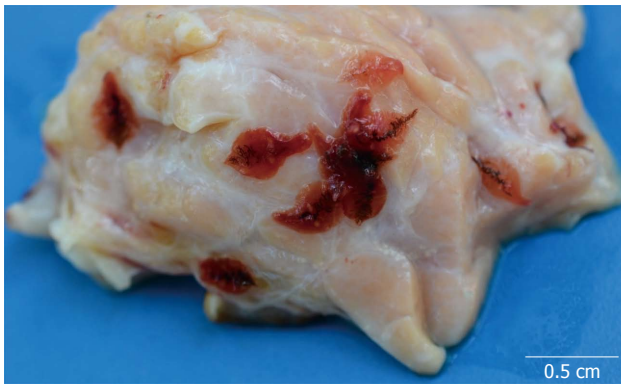


**Figure 2 *Eurytrema* sp. in bovine pancreas.** The fluke is red, oval shaped and measuring between 8 to 13 mm in length by 6 to 7 mm width. The pancreas of infected animals has a harder consistency and hyperplasic ducts partially filled with adult parasites are observed.

triclabendazole did not present efficacy.

There have been only a few *in vivo* trials aiming to control the disease, and at the moment there are no treatment protocols establishing an appropriate chemotherapy. Even if there are some apparently efficient antiparasitics, the treatment of an infection cannot be accomplished in Brazil. There are no available products containing praziquantel authorized by the regulatory agencies to be administrated in cattle in Brazil, while nitroxinil, according to the current legislation, cannot be applied in dairy cows. Therefore, these two factors make the treatment of Eurytrematosis impossible in dairy cattle in Brazil, as well as in many other countries.

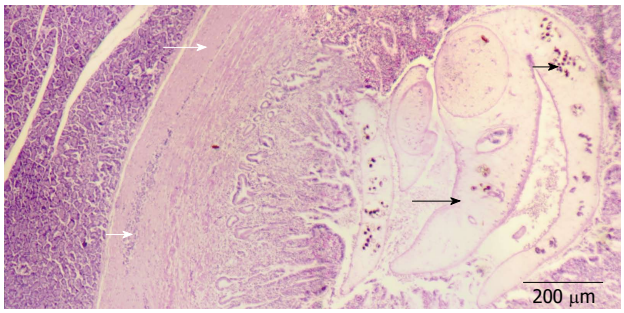


**Figure 3 Histopathology of pancreas with Eurytrematosis.** Moderate proliferation of connective tissue (white large arrow), severe hyperplasic ducts containing adult parasites (black large arrow), parasite eggs (black short arrow) and mild inflammatory infiltrates of lymphocytes, eosinophils, plasma cells and macrophages (white short arrow). The islets of Langerhans are usually not affected but in most severe cases, they are replaced by connective tissue.

With the aim to control the disease, our group is developing many studies: (1) Molecular determination of the *Eurytrema* species that occurs in cattle and sheep in our region; (2) Establishment of the effectiveness of antiparasitic drugs against *Eurytrema* sp. strains presented in the west of Santa Catarina, Brazil. For this, dairy cattle herds will be segregated into groups, and each group will be treated with a different molecule, taking into account the main drugs used in the region and with known action against trematodes. The second aim of this work is to evaluate if the sub-clinical damage in the pancreatic parenchyma indirectly lead to economic losses; (3) Measure the oxidative stress caused by the parasitism. This comprises gauging the parameters that indicate cellular lesions and the antioxidant status in the pancreas. There are many studies that correlate oxidative stress with clinical-pathologic findings and pathogenesis in infectious diseases. The consequences of infection by *Eurytrema* sp. to an animals' health and productivity have not been elucidated and articles about the oxidative stress in animals parasitized by *Eurytrema* sp. are not available; and (4) Another future study will reproduce the parasites' complete life cycle in the laboratory, aiming to find possible immunogens in the newly encysted juveniles stage of *Eurytrema* sp.

The results of our studies will contribute to elucidate the real importance of Eurytrematosis to animal health and production in Brazil. In the long term, we expect to contribute with directly applicable results to both agribusiness and public health. It is necessary to determine protocols capable of controlling Eurytrematosis in an efficient and viable way. Productivity losses may also be estimated to determine the real economic impact and importance of the disease. We also expect to find different ways to apply a biological and/or immunoprophylaxis control against this parasite, as chemical therapy alone is typically insufficient for the control and prevention of many illnesses, both in

veterinary and human medicine.

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REVIEW

# Use of siRNA molecular beacons to detect and attenuate mycobacterial infection in macrophages

Remo George, Renata Cavalcante, Celso Carvalho Jr, Elyana Marques, Jonathan B Waugh, M Tino Unlap

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Author contributions: George R, Cavalcante R, Carvalho Jr C, Marques E, Waugh JB and Unlap MT equally contributed to this work; George R, Waugh JB and Unlap MT designed the research; George R, Cavalcante R, Carvalho Jr C, Marques E and Unlap MT performed the research; Waugh JB and Unlap MT analyzed the data; George R, Waugh JB and Unlap MT wrote the paper.

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## Abstract

Tuberculosis is one of the leading infectious diseases plaguing mankind and is mediated by the facultative pathogen, *Mycobacterium tuberculosis* (MTB). Once the pathogen enters the body, it subverts the host immune defenses and thrives for extended periods of time within the host macrophages in the lung granulomas, a condition called latent tuberculosis (LTB). Persons with LTB are prone to reactivation of the disease when the body's immunity is compromised. Currently there are no reliable and effective diagnosis and treatment options for LTB, which necessitates new research in this area. The mycobacterial proteins and genes mediating the adaptive responses inside the macrophage is largely yet to be determined. Recently, it has been shown that the *mce* operon genes are critical for host cell invasion by the mycobacterium and for establishing a persistent infection in both *in vitro* and in mouse models of tuberculosis. The YrbE and Mce proteins which are encoded by the MTB *mce* operons display high degrees of homology to the permeases and the surface binding protein of the ABC transports, respectively. Similarities in structure and cell surface location impute a role in cell invasion at cholesterol rich regions and immunomodulation. The *mce4* operon is also thought to encode a cholesterol transport system that enables the mycobacterium to derive both energy and carbon from the host membrane lipids and possibly generating virulence mediating metabolites, thus enabling the bacteria in its long term survival within the granuloma. Various deletion mutation studies involving individual or whole *mce* operon genes have shown to be conferring varying degrees of attenuation of infectivity or at times hypervirulence to the host MTB, with the deletion of *mce4A* operon gene conferring the greatest degree of attenuation of virulence. Antisense technology using synthetic siRNAs has been used in knocking down genes in bacteria and over the years this has evolved

into a powerful tool for elucidating the roles of various genes mediating infectivity and survival in mycobacteria. Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is rapidly gaining popularity.

**Key words:** Mammalian cell entry; Molecular beacons; siRNA; Mycobacterium tuberculosis; Macrophages



**Core tip:** This review paper looks at the current status of research of the role of mammalian cell entry gene products in mediating cholesterol mediated latency of mycobacteria and the potential use of short-interfering RNA molecular beacons in detecting and attenuating mycobacterial infections.

George R, Cavalcante R, Carvalho C Jr, Marques E, Waugh JB, Unlap MT. Use of siRNA molecular beacons to detect and attenuate mycobacterial infection in macrophages. *World J Exp Med* 2015; 5(3): 164-181 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i3/164.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i3.164

## EPIDEMIOLOGY OF LTB

The year 2005 marked the 100$^{th}$ anniversary since Robert Koch received the Nobel prize for his work on tuberculosis (TB) and yet more than one hundred years later the World Health Organization (WHO) has reaffirmed its designation of TB as a global emergency[1]. Tuberculosis still remains a pandemic, infecting one-third of the world's population and killing millions of people each year. Estimates are that a TB death occurs every minute. According to recent estimates of WHO, nearly 9 million people were infected with TB in 2012, including 1.3 million TB-related deaths worldwide[2]. Incidentally, TB and reactivation of latent TB have turned out to be the leading causes of death for people who are infected with human immunodeficiency virus (HIV). At the same time, according to the Centers for Disease Control (CDC), there were 9945 TB cases reported in the United States in 2012[3]. More than 80% of TB cases in the United States are from reactivation of latent TB infection[4].

Tuberculosis is a disease that spreads from person to person through the air and is mediated by the pathogen *Mycobacterium tuberculosis* (MTB). The TB bacillus was discovered in 1882 and has been the subject of extensive research since then. There is still much to be learned about the nature of this organism, its virulent properties, and its response to host defenses. TB affects the lungs mainly, but can also have other target organs such as brain, spine and the kidneys. When a person with TB infection coughs or sneezes, droplets containing MTB are released into the air and when another person breathes in the infected droplets, they can be infected. However, not everyone infected with TB bacteria becomes sick. There are two TB-related conditions that exist: latent TB (LTB) infection and active TB disease. Those that have latent TB infection do not feel sick or do not present with any symptoms. In LTB cases, even though they are infected with the mycobacterial pathogen, they do not have active TB disease. Overall, it has been shown that about 90% of the people infected with MTB will have LTB infection and 10% will eventually go on to have full-blown active TB at a later stage in their life[5]. Nearly 50% of those who develop TB do so within the first two years of infection. This rate is even higher in immunocompromised individuals, such as those with HIV infection, where the risk of developing TB from LTB activation is significantly higher. Also of particular concern are those infected with drug-resistant TB (XDR TB)[3].

Thus, this ancient human adversary continues to be a challenge in all aspects of medical care, from prevention to diagnosis and therapy.

## NEED FOR IMPROVED DIAGNOSTIC AND THERAPEUTIC METHODS FOR LTB

Currently, there are no tests available to directly detect *in vivo* the presence of latent MTB in an affected individual and assessment of latent infection involves an imperfect approach of measuring the host immune response to mycobacterial infection[4]. On the contrary, active TB infection is diagnosed by detecting MTB bacteria in clinical samples taken from patients. A positive diagnosis can be made only by culturing MTB from the specimen, even though the results from this may take four to eight weeks for conclusive answers. Other methods for diagnosing TB include chest X-rays, patient sputum smear microscopy, polymerase chain reaction (PCR) testing, immunological memory-based tests including the less specific purified protein derivative (PPD/tuberculin) skin test and more specific Interferon-γ release assays, phage amplification assays, solid and automated liquid cultures, as well as several tests for antibiotic resistance. These tests can only strongly suggest the presence of active TB or LTB as a diagnosis but they cannot confirm the presence of the bacteria in the body. Reliable and rapid diagnosis of latent TB is a major challenge in low socioeconomic areas, and even in parts of developed countries, especially in areas where immunodeficiency diseases like acquired immune deficiency syndrome are more endemic. In many cases, patients have to undergo time-consuming multiple testing before reaching an apparent diagnosis. This testing deficiency can be especially critical when trying to identify high-risk individuals for prophylactic regimen, and also for identifying and managing extrapulmonary TB sites in HIV co-infected patients[6].

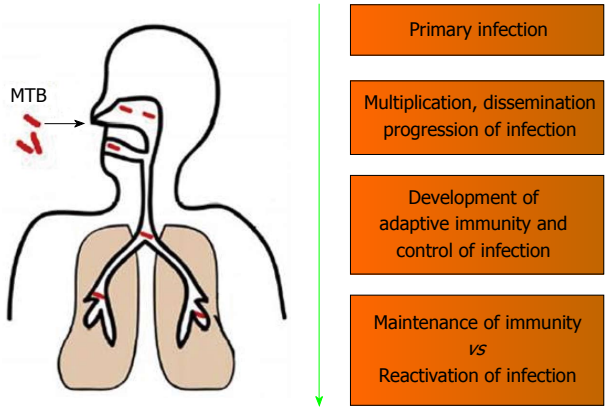


**Figure 1 Spectrum of host immune responses against *Mycobacterium tuberculosis*.** MTB: *Mycobacterium tuberculosis*.

Treatment for active TB cases consists of a combination of four first-line antibiotics for a period of two months, followed by two drugs for another four months[7]. First line antibiotics consist of rifampicin, isoniazid, pyrazinamide, and ethambutol. These drugs are effective mainly in actively dividing bacilli and its effectiveness in treating LTB, where the bacilli are dormant, has not yet been proven. The treatment of LTB is usually long term with the intent of sterilizing the non-replicative or slowly replicating bacteria[8]. If the particular mycobacterial strain is resistant to the first line drugs, then treatment is escalated for up to 18 mo with five lines of available drugs. Surgery is also performed as a last resort if treatment fails due to drug resistance. Since the chemotherapy regimen for active and latent TB infections usually spans many months, poor patient compliance rates is a major issue contributing to the emergence of resistant strains[4]. There is a pressing need for rapid and inexpensive tests to confirm latent TB cases in order to manage this global epidemic.

Developing a direct MTB imaging screening tool for the asymptomatic population along with novel treatment strategies is vital to our fight against TB. This is especially true for high-risk categories with LTB such as drug users with unsanitary needles, healthcare workers in high risk and densely populated environments, the medically under-served poor and minority populations, children exposed to high risk adults, immunocompromised patients and patients on immunosuppressant drugs, and health care workers who serve these high risk populations[9]. Developing a direct MTB imaging screening tool with combined therapeutic applications for the asymptomatic population is going to have vitally important and far reaching impact in the fight against TB.

## SPECTRUM OF HOST IMMUNE RESPONSE AGAINST MTB AND DEVELOPMENT OF LTB

Humans are the only natural host of MTB and are highly susceptible to MTB infections. Even a few (5-10) bacilli is capable of mediating a primary infection[10]. The initial interaction of the MTB with the host involves alveolar macrophages, which is the only known cell type to harbor MTB *in vivo*[11]. Upon coming in contact with MTB, the interaction of the host immune response with MTB can be divided into 4 general types of events[12]: (1) Primary infection event involving the invading MTB; (2) Events that would promote the dissemination and progressions of the MTB infection; (3) Development of an adaptive immunity that would lead to the containment MTB infection; and (4) Interplay of protective immunity involved in latency *vs* immunologic compromises leading to reactivation of MTB infection (Figure 1).

Phagocytosis of the MTB by alveolar macrophages followed by its intracellular growth initiates the cascade of immune events of the primary infection[13]. Activation of the components of the innate immunity, the recruitment of various classes of monocytes and lymphocytes to the site of infection, and the final development of specific immunity allow for the containment of infection (Figure 2).

The hallmark of latent TB is the granulomatous lung parenchymal lesions and their draining lymph nodes which is called the "Ghon complex". The events leading to the formation of granuloma begins when the MTB is inhaled into the lungs and the bacterium is phagocytosed by alveolar macrophages and dendritic cells. The infected cells release proinflammatory cytokines that help recruit more immune cells to the site of infection. The cytokines IL-12 and IL-18 from the infected cells induce natural killer cell activity, which in turn produce IFN-$\gamma$ that help active macrophages to produce tumor necrosis factor-$\alpha$ and other micobicidal substances. Through the actions of these cytokines and chemokines, other immune cells are recruited leading to the formation of the granuloma[14,15]. In the granuloma, the macrophages further differentiate into epitheloid cells and foamy macrophages and are surrounded by lymphocytes and an outer layer of fibroblasts and matrix proteins. The morphology of the lung granuloma is characterized by a central necrotic core surrounded by concentric layers of macrophages, epitheloid cells, multinucleated Langhans giant cells, and lymphocytes[16,17]. Containment of MTB at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence (Figure 3). Successful containment of the pathogen to the site of the primary lesion results in latent infection, which appears in chest X-rays as calcified granulomatous lesions[18].

The exact location of dormant MTB organisms in latent TB has not been elucidated[18]. Studies have shown evidence of the presence of the pathogen in the normal tissue surrounding the granuloma necrotic centers[19] which appears to be the preferred location of the pathogen during latency[20].

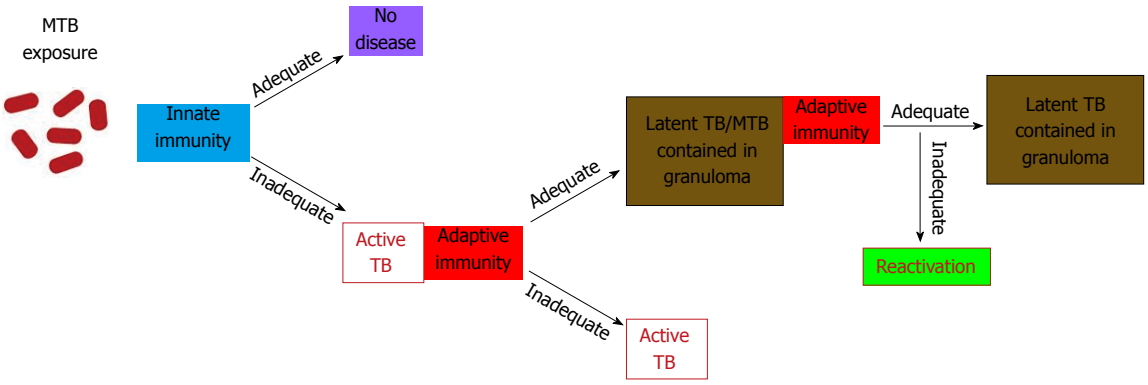


**Figure 2 Latent tuberculosis pathogenesis and transmission profile.** Most people are adequately protected by innate immunity against MTB infection, however, the infection progresses when the innate immune response levels are inadequate. The development of specific immunity leads to the containment of MTB in granulomas as asymptomatic latent infection. However, inadequate adaptive response at any time progresses to the reactivation and development of full-blown active tuberculosis. MTB: *Mycobacterium tuberculosis*.

A dynamic balance between the host immune response and the MTB pathogen is maintained during latency. Direct cross-talk between MTB and the host immune response occurs in a dense region surrounding the granuloma which is derived from lymphocyte infiltration[21,22]. The granuloma has a central necrotic core which serves as nutritional source for persisting mycobacteria, surrounded by the thick leukocyte wall which prevents the spread of the mycobacteria. The leukocyte derived wall surrounding the granuloma is highly vascularized and facilitates the delivery of drugs against latent TB[23].

## POST-PHAGOCYTIC MOLECULAR EVENTS FOLLOWING MYCOBACTERIAL ENTRY

Once entering the host, the ability of MTB to survive decades within the body of the host by subverting the host immune defenses is of continued intrigue and fascination. The precise mechanism of how the bacteria is able to achieve this long term dormancy leading to LTB is still unknown, however, recent advances in mycobacterial molecular biology have shed some light into these processes.

Macrophages make up the major component of the innate host defense, and they do this by pathogen recognition, ingestion and killing of foreign microbes that enters the body including pathogenic and non-pathogenic mycobacteria. The pathogenic mycobacteria have developed a number of strategies to subvert the host immune defenses and evade the destructive action of the macrophages, eventually surviving within this normally inhospitable cell for long periods of time thus resulting in the disease[24]. The surviving bacteria within the macrophages can be in a latent state with stationary growth or, given the right conditions in an immunocompromised host, can switch to a metabolically active state that facilitates proliferation, dissemination and active disease.

Upon gaining entry into the body, most non-pathogenic microbes get phagocytosed by the macrophage into a phagosome where the invading microbe gets exposed to high levels of reactive oxygen species and reactive nitrogen species. The phagosome then goes on to mature and fuse with the organelles of the endocytic pathway, thereby acquiring surface molecular markers which leads to the acidification of the phagosome to pH 5 as well as gaining hydrolytic enzymes that digest the invading microbe[25,26] (Figure 4A). MTB, however, has developed several ways to evade attack by the macrophage and creates a favorable environment for replication (Figure 4B). This is mainly by inhibiting several aspects of phagosomal maturation, including fusion and fission events along the endocytic pathway and the recruitment of vacuolar $H^+$-ATPases[27,28]. The MTB carrying phagosome retains characteristics of an early phagosome with regard to its pH (about 6-6.5), presence of Rab5 (a Rho-GTPase directing endosomal trafficking and mediating fusion between phagosomes and other organelles), and continued access to other recycling endosomes[29,30], but it lacks mature hydrolases[31] and cathepsins, with interactions between the phagosome and the *trans* Golgi Network blocked[32]. Several MTB products are believed to be inhibitors of phagosomal maturation, including components of the mycobacterial cell envelope such as lipoarabinomannan, trehalose dimycolate and sulpholipids, phosphatase SapM and kinase PknG[29], and the secreted protein ESAT-6[33]. The exact mechanism behind the inhibition of phagosomal maturation by the mycobacteria is yet to be elucidated.

Studies have shown that cholesterol is a necessary component for the uptake of the MTB into the macrophage *via* the complement receptors and for the inhibition of phagosomal maturation[34] (Figure 4B). A host protein associated with the cell membrane called tryptophan-aspartate containing coat protein (TACO) is recruited and retained in the phagosomes harboring mycobacteria thereby preventing the bacterial delivery to lysosomes[35]. TACO is an actin-binding protein seen associated with cholesterol rich regions of the host macrophage plasma membrane[34]. The mycobacterium

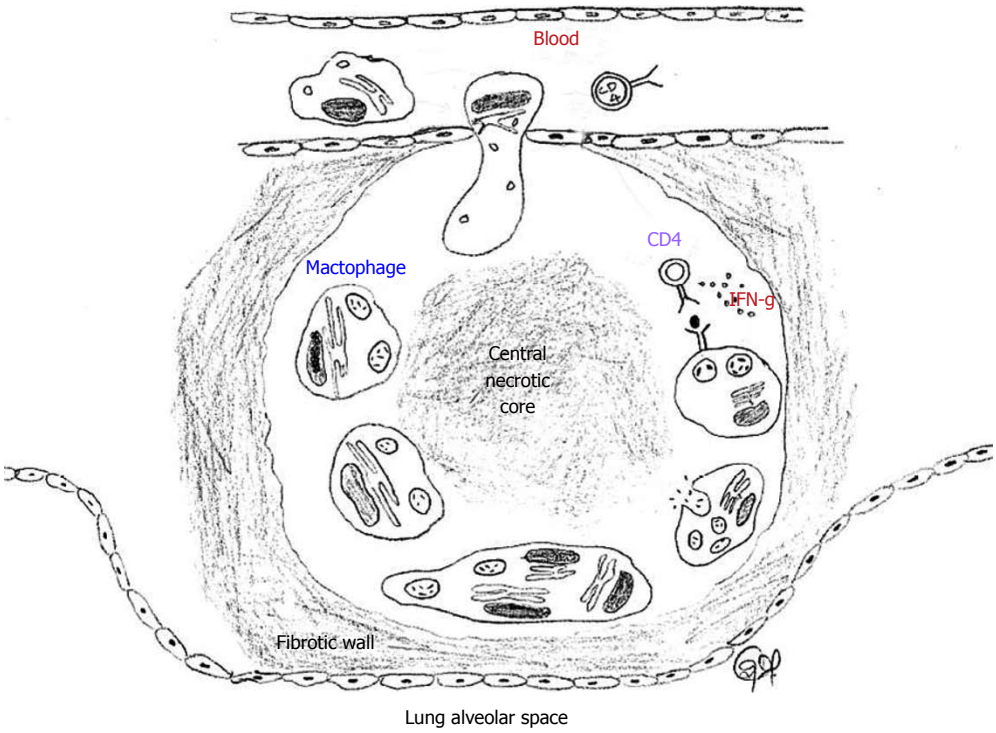


**Figure 3 Tuberculosis granuloma.** A granuloma sequesters MTB infected macrophages and is surrounded by immune cells, predominantly $CD4^+$ helper T lymphocytes. Some infected macrophages fuse to form foamy giant cells. The infected macrophages and giant cells present antigens to T cells and activate them to produce a variety of cytokines and chemokynes, and also kills the infected macrophage and the MTB. The chemokines also serve to recruit additional T cells to the granuloma from the circulating blood. IFN-γ activates the macrophages to kill the intracellular MTB by generating reactive oxygen species and reactive nitrogen species intracellularly. The center of the granuloma is filled with cell debris and both live and dead MTB spilled from dead macrophages (caseation), all of which form a central hypoxic necrotic core. A sheath of collagen fibers produced from lung fibroblasts surround the granuloma. MTB: *Mycobacterium tuberculosis*.

within the phagosome is somehow able to prevent the removal of the TACO coat protein which prevents the fusion of phagososome with lysosome[35]. Moreover, it has been shown that TACO-mediated uptake of mycobacteria depends on cholesterol[34,36,37]. The mycobacterial proteins and genes mediating these adaptive responses inside the macrophage is largely yet to be determined. MTB′ unique ability to utilize cholesterol, a component of cell membranes, also plays a role in its persistence[38]. In the nutrient-deficient intracellular environment, MTB adapts its metabolism by alternating between carbohydrate and fatty acid metabolism[39]. Studies have shown that MTB utilizes cholesterol for its energy needs and for the biosynthesis of virulence-associated lipid phthiocerol dimycocerosate[38]. A number of reports indicate that MTB metabolizes cholesterol during host infections and the metabolic products contribute to the long-term survival of MTB in the host[38,40,41]. Furthermore, because the cholesterol catabolism pathway requires a large number of oxygenases, it should be no surprise that MTB infects the lungs where oxygen concentration is the highest[42].

## *MCE* OPERONS AND THEIR ROLE IN MYCOBACTERIAL INFECTION AND PERSISTENCE

Over the recent years, it was shown that a DNA fragment from MTB cloned into *Escherichia coli (E. coli)* could mediate the latter's entry and survival in mammalian cells[43] and was named as the mammalian cell entry (*mce*) operon. The *mce* operon genes have been shown to be important in the invasion of the mammalian host cell by the mycobacterium and for establishing a persistent infection both *in vitro* and in mouse models[44,45]. The analysis of the complete genome sequence of MTB in 1998[46] showed that the *mce* operon is composed of a group of four homologous *mce* operons (*mce1, mce2, mce3*, and *mce4*). It was found that all the constituent *mce* genes in the four operons were arranged in an identical manner. Each of the operon contained eight genes, of which two genes preceding the *mce* genes are named *yrbEA* and *yrbEB* which encoded for integral membrane proteins and the six *mce* genes potentially encoding exported proteins (secreted or surface-exposed) thought to be important for the entry and survival of the pathogen in the mammalian cells[46]. The four *mce* operons are widely seen throughout the genus Mycobacterium[34] and the general organization of the genes in each of the four operons are shown in Figure 5.

The YRBE and MCE proteins encoded by the MTB *mce* operons have structural homology to the permeases and the surface binding protein components of the ABC transporters, respectively[47]. The typical ABC permease contains six trans-membrane helices with the C-terminus located on the cytoplasmic side of the membrane

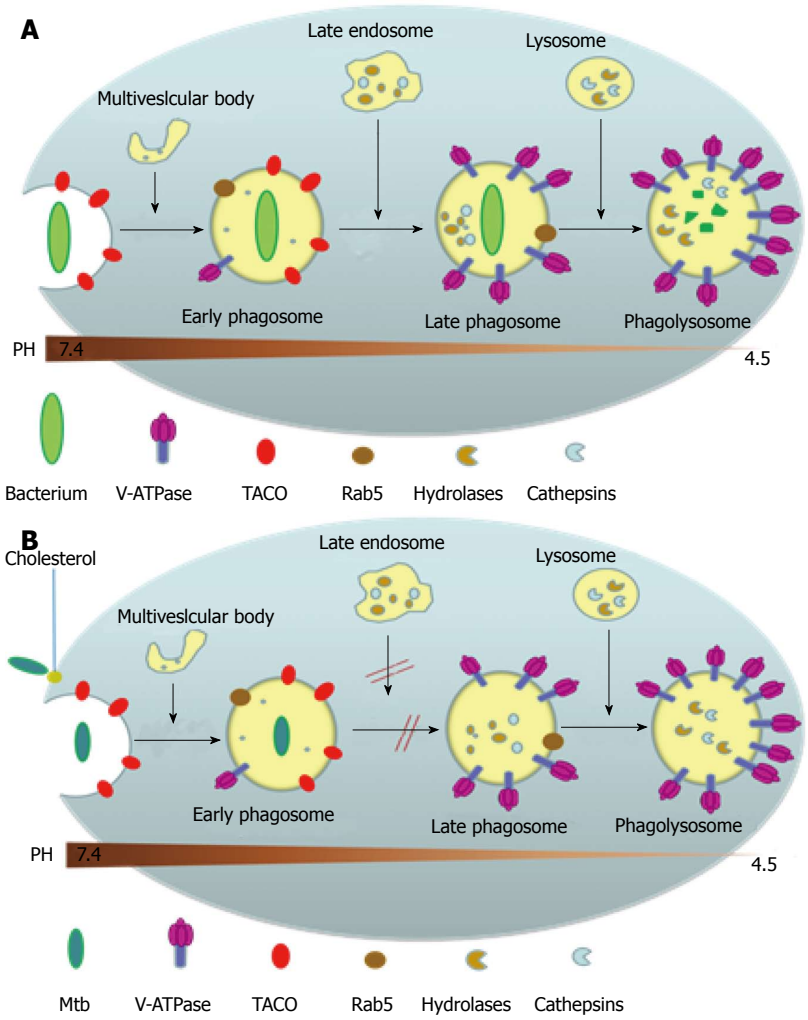


**Figure 4 Phagosomal maturation or arrest following pathogen uptake (A) or *Mycobacterium tuberculosis* uptake (B), respectively.** (A) shows that upon entering into the body, most non-pathogenic microbes are phagocytized by the macrophage into a phagosome which then goes on to mature by fusing with the vesicles of the endocytic pathway and to finally fuse with lysosomes. These phagosomes undergo acidification due to the presence of proton-ATPase molecules from vacuolar membranes and the lysosomes, and this increased level of acidification activates the lysosomally derived acid hydrolases, cathepsins and other enzymes, along with reactive oxygen and nitrogen intermediates, to destroy the pathogen. Phagocytosis also initially triggers the recruitment of TACO around the particle to be ingested, as a result of the latter's initial association with cell cortex microtubules, but is released prior to the lysosomal delivery of the bacteria; (B) Shows the effect of *M. tuberculosis* on phagosome maturation. Cholesterol serves as a docking site for the mycobacteria and its cell surface receptor there by facilitating its phagocytosis at cholesterol-rich regions. Cholesterol plays a crucial role in not only the entry of mycobacteria into macrophages but also mediates the phagososomal association of TACO (Coronin 1), a coat protein associated with cholesterol-rich regions which is actively retained on the phagosomal membrane housing the mycobacteria through a yet unknown mechanism, which prevents the degradation of the mycobacteria in the lysosomes. TACO: Tryptophan-aspartate containing coat protein.


**Figure 5 The organization of the mce operons.** The operon structures of the four mce operons with their constituent genes are shown. The grey arrows represent yrbE genes and the blue arrows represent the mce genes.

(Figure 6A). The YRBE permease contains five or six transmembrane segments outside the C-terminus and the orientation of the N-terminal transmembrane helix may be either cytoplasmic or outside (Figure 6B), suggesting a transmembrane transport role[47,48].

The MCE protein, including MCE1A, 3A and 4A, but not MCE2A, make up a patch of 275-564 amino acid residues, with the hydrophobic stretch at the N-terminal anchored in the membrane, after folding and modification[47], along with a 22 amino acid "invasion domain" near the C-terminal exposed outside the membrane[49,50] (Figure 6B). These characteristics are consistent with their cell surface location and proposed role in cell invasion and immunomodulation[51], however,

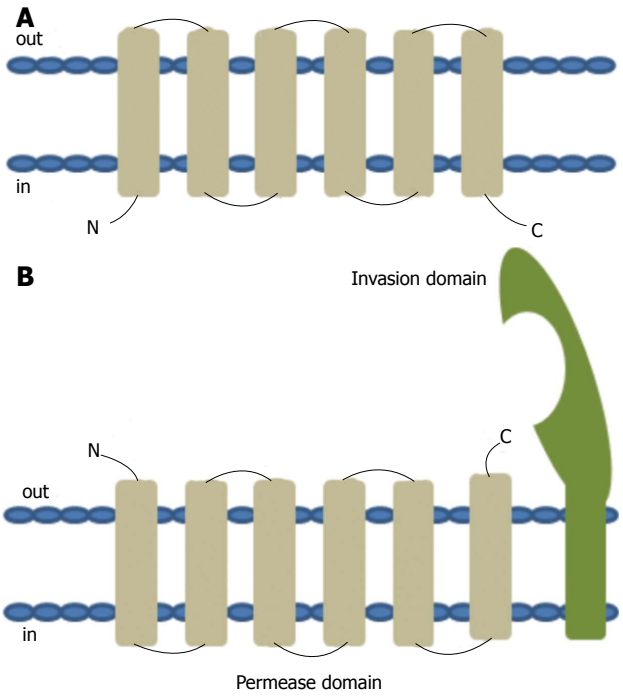


**Figure 6 Topology of typical ATP binding cassette transmembrane permease (A) and the predicted topology of YRBE permease and MCE invasion domain (B).** ATP binding cassette (ABC) transport systems in both prokaryotes and eukaryotes consists of four domains, two cytoplasmic ABC domains and two hydrophobic membrane spanning permease domains (A). Each of the membrane spanning domains typically contains 6 hydrophobic transmembrane segments anchored in the cell membrane with the C- and N-termini located towards the cytoplasmic side. ABC transporters in prokaryotes that function as importers also typically require additional extracytoplasmic helper proteins called 'substrate binding proteins' for function. Each of the mce operons contains two YRBE domains that have structural homology to the permease domain of the ABC transporters (B). Each YRBE permease contains five or six transmembrane segments with the C-terminal end located on the extracytoplasmic side and the N-terminal end being either cytoplasmic or extracellular. The MCE protein, MCE4A, consists of a patch of 400 amino acid residues, with a hydrophobic stretch anchored in the cell membrane and a 22-amino acid invasion domain exposed outside to the membrane, which is predicted to be structurally similar to the substrate binding domain of ABC importers.

the mechanism of interaction between YRBE and MCE proteins is not yet clear[52].

Phylogenetic studies have shown that the MCE proteins share between 30%-70% amino acid identity to their inter-operon counterparts but only 16%-26% identity with other MCE proteins encoded by the same operon. For example, there is 61% identity between MCE1A and MCE2A, however, it falls to 25% identity between MCE1B and MCE1C[53,54]. Because of the multiple *mce* operons in the genome, it is proposed that they may have redundant or time dependent activities. This possibility is supported by the temporal transcriptional expression differences during different stages of *in vitro* growth between *mce1* in comparison to *mce3* and *mce4*[55]. Differences are also seen with *in vivo* growing bacilli in that even though *mce1*, *mce3*, and *mce4* transcripts are detectable up to 24 wk post infection in rabbit lung tissue, only *mce4* transcript is detectable 16 wk post infection in guinea pig spleen[55]. The expression of *mce2* was not detected under any conditions tested[55].

Studies using *mce* operon deletion or disruption mutants of MTB have demonstrated varying effects with the different *mce* operons. Some studies have shown than disrupting *mce* operons lead to attenuation[56,57], while others have shown some degree of hypervirulence for the host MTB following the mutations[58,59]. Deletion of *mce* operons 3 and 4 attenuated MTB virulence in infected macrophages[60].

The *mce4A* gene is the first among the six *mce* genes in the *mce4* operon that is studied the most. Studies showed that the MCE4A protein is not only important for host cell invasion but also for survival of the MTB pathogen in human macrophages[61,62]. Individual *mce1*, *mce2*, *mce3*, and *mce4* mutants administered intranasally or intravenously in mice have shown to result in lower bacterial burdens and slower mortality of the infected mice, with *mce4* operon deletion showing the greatest effects on MTB virulence[56,60]. The route of infection was also shown to be having an effect on the attenuation results in one study[56]. Hypervirulence among *mce1* mutants have been demonstrated in two separate studies when administered intranasally, intravenously, or intraperitoneally[58,59]. The deletion mutants in all the above studies, however, were not identical in the nature of their deletions and had variations in their deletion sequences that possibly led to different polar effects on downstream genes, which may explain the discrepancy in the results of some of the deletion mutant studies.

Apart from its possible role in mediating host infection, it is thought that at least some of the MCE4 proteins form an outer membrane channel that mediates cholesterol entry into the cell, thereby enabling uptake by the mycobacterium of host lipids vital for its survival during the prolonged latent infection[63]. Transposon Site Hybridization studies have shown that certain *MTB* genes involved in the lipid metabolism was genetically linked to the *mce4* operon genes[60]. The *mce4* has been shown to encode a cholesterol transport system that enables the mycobacterium to derive both carbon and energy from the host membrane lipids and also for possibly generating sterol metabolites mediating its long-term survival within the macrophage[38], with the *mce4* expression progressively increasing as the latency phase advances[64].

## ANTISENSE TECHNOLOGY IN BACTERIA

Hundreds of bacterial encoded short interfering RNAs (siRNA) have been reported over the past decade[65-67]. Majority of these siRNAs act by binding to their target mRNAs to bring about the repression. They fall into two major categories: some are encoded at locations farther away from the target gene (trans-acting) and others are encoded by DNA strand complementary to the target gene (cis-acting). The trans-acting siRNAs

generally share only limited complementarity with their target gene and thus is prone to have off target effects. Trans-acting siRNAs are by far the most characterized bacterial siRNAs and have been shown to usually require the chaperon protein Hfq for base pairing[68]. The cis-encoded siRNAs, or anti-sense RNAs, have perfect complementarity with their target gene and thus have more extensive and stronger base pairing. Among the reported bacterial antisense RNAs, some are short (siRNA), with around 100 nucleotides in length and are usually encoded by plasmids or bacteriophages, while some are chromosomally encoded and are longer, in some cases overlapping entire genes or corresponding to the 5′ or 3′ extension of the protein coding region of the mRNA. The 5′ untranslated region of the *mogR* mRNA in *Listeria monocytogenes* overlaps 3 genes on the opposite strand involved in the flagellar synthesis and serves as an example of chromosomally encoded long antisense RNAs[69]. The binding region of antisense RNA on the target mRNA can also vary and may be located in the 5′ end, 3′ end, the central region, or the entire coding region.

Antisense RNAs in the bacterial cell have been shown to repress many detriments to the cell such as transposons and toxic proteins. One of the first anti-sense RNA to be discovered in bacteria was the RNA-OUT of the transposon Tn10, which was shown to inhibit transposition by preventing the translation of transposase mRNA[70]. Antisense RNAs are also seen encoded opposite transposase genes in *Salmonella enterica*[71,72], *Caulobacter crescentus*[73], and *Listeria monocytogenes*[69]. Thus a critical role of bacterial antisense RNA, as in eukaryotes[74], appears to be the inhibition of transposition. There is increasing evidence that antisense RNAs downregulate the expression of toxic proteins[75,76]. It has been found that most of these repressed proteins are hydrophobic, small with less than 50 amino acids, and toxic at higher levels. An example of this tight repression of one such toxic protein is seen in *E. coli*, where the low levels of SymE protein is maintained by the LexA repressor of the save our souls response, the SymR antisense RNA and the Lon protease[77]. Some of the antisense RNAs to transposases and toxic genes, such as SymR, are expressed constitutively in the cell[77].

Studies have shown that antisense RNAs can positively and negatively regulate the expression of various transcriptional regulators and other metabolic and virulence proteins in bacterial systems. For example, The 109 nucleotide *GadY* antisense RNA of *E. coli* overlaps the intergenic region of the dicistronic *gadXW* mRNA which encodes two transcription regulators of the acid stress response genes and enhanced transcription of the *GadY* gene which leads to cleavage of *gadXW* mRNA into *gadX* and *gadW* transcripts, leading to positive regulation (increased expression) of those genes[78,79]. And on the other hand, in the nitrogen-fixing cyanobacterium *Anabaena sp.* PCC 7120, the approximately 2200 nucleotide *alr1690-α-furA* antisense RNA spans the entire *alr1690* coding region and extends through the gene encoding the ferric uptake transcriptional regulator, FurA, into its promoter and regulator regions and it helps decrease *furA* expression and translation, thereby acting as a negative regulator of iron absorption and nitrogen metabolism[80]. Similar regulatory RNAs controlling metabolic responses to environmental effects have been reported in many other bacterial systems[81-87].

Another recently discovered phenomenon is the antisense-mediated gene regulatory switch in the bacteria called the "excludon". This comprises a gene locus encoding an unusually long antisense RNA that spans divergent genes or operons with related or opposing functions. In such a regulatory system, the antisense RNA can inhibit the expression of one operon while functioning as an mRNA for the adjacent operon, there by acting as fine-tuning regulatory switches in bacteria[88].

Antisense RNA also regulates the expression of various structural and virulence factors in different bacteria. For instance, the 1200 nucleotide *AmgR* RNA which is encoded opposite the *Salmonella enterica mgtCBR* operon is responsible for the bacteria's virulence and survival in macrophages[89]. A number of other antisense RNAs modulating virulence and regulating host-pathogen interactions have been discovered in a variety of bacterial species over the years[90-97]. Antisense RNAs have also been found to impact other benign structural components including flagellar synthesis in *Rhizobium*[98], *H. pylori*[99], *L. monocytogenes*[69] and *S. enterica*[100].

The first complete experimental confirmation of short antisense RNAs in mycobacteria was published in 2009, which revealed 5 trans-acting and 4 cis-acting siRNAs in MTB H37Rv in the context of pH and oxidative stress[101]. By the end of 2013, a total of more than 200 endogenous antisense RNAs were experimentally identified in various mycobacteria, including 70 in MTB[102-110], 90 in *M. Bovis*[103,104,111], 9 in *M. avium*[112], and 44 in *M. smegmatis*[102-104,113]. From these recent studies, a stronger connection between mycobacterial pathogenesis and the levels of expression of the antisense RNAs have emerged but many new questions about their potential pathogenic *vs* housekeeping functions remain to be answered. The lack of identification of an Hfq homolog in mycobacteria prevents the current approach of coimmunoprecipitation, making the study of the role of antisense RNAs all the more difficult in this genus[102]. Pandey *et al*[114] have proposed an alternative protein, Rv2367, as a potential RNA chaperon in place of Hfq[114], however, studies are ongoing in this direction to find a functionally-equivalent chaperon or to get around this issue[113]. Also, the role of mycobacterial antisense RNAs in regulating transposition is not as clear as in other bacterial systems like *E. coli*.

Antisense RNAs can repress or modulate expression of target genes by a variety of mechanisms, including transcription interference, transcription attenuation,

degradation by endo- or exonucleases, or by blocking ribosome binding. When inducing transcription interference, the transcription of antisense RNA from one promoter hinders the RNA polymerase from either binding or extending the target gene transcript from the opposite strand[115,116]. This type of interference occurs only in Cis and does not involve base pairing. In transcription attenuation, the binding of the antisense RNA to the target RNA causes a conformational change creating a terminator structure in the mRNA leading to its premature termination of transcription[117]. Antisense RNAs can affect target mRNA stability by stimulating or inhibiting its degradation. When employing endo- or exonucleases for gene regulation, the antisense RNA, upon binding to its target mRNA, induces or blocks a ribonuclease target site within the mRNA or can indirectly block the binding of the ribonuclease at a distant site. In many bacterial systems, two major endoribonucleases have been identified, RNase Ⅲ which cleaves double stranded RNA into two with different stabilities than the original transcript[118-121], and RNase E, a component of the multi-protein degradasome complex which cleaves single stranded RNA and interacts with Hfq and globally affect mRNA stability[119,122]. It is not precisely clear how the antisense RNA modulates RNase E activity, but the proposed mechanisms include the donation of its 5′ monophosphate to stimulate RNase E activity or physically block the RNase E recognition site by basepairing to downregulate activity[123]. Other ribonucleases in bacteria have also been identified with more specialized functions, including RNase G (a non-essential paralog of RNase E)[124,125], RNase P[126,127], RNase LS[128], RNase Z[129,130], RNase H[131], RNase J1/J2[132], and the recently characterized RNase Y[133]. Many of these ribonucleases have already been characterized in various Mycobacteria, including MTB and *M. smegmatis*[134-142]. Apart from these mechanisms, it has also been found that some antisense RNAs can physically block mRNA expression by binding to the Shine Dalgarno sequences of their target mRNA and prevent ribosome binding[66,77,80], or they may indirectly modulate expression by altering the target mRNA conformation[67]. Finally, antisense RNAs can exhibit dual functions by acting as mRNAs and antisense RNAs or cis- and trans- acting RNAs[78].

Two general mechanisms have been proposed for base-pairing in antisense RNAs. The first type is a single-step mechanism in which the antisense RNA makes initial contact with the target mRNA to form a duplex[143]. The second type is a multi-step system in which the initial duplex formed is stabilized by a protein, followed by the formation of the more stable complete duplex[144,145]. In many cases of base pairing for the antisense RNA, a stem-loop structure is found to be important, along with a "pyrimidine-uracil-any nucleotide-purine" U-turn motif[146].

Synthetic antisense RNAs are generally delivered either by expressing the antisense transcript from a gene introduced into the cell or by direct delivery of antisense oligonucleotides. Degradation of the antisense transcripts can be a problem for both these delivery approaches, however, this issue is mitigated by using sequences that form more stable hair-pin structures with paired ends[147]. In order to increase the stability and uptake, antisense RNAs have been modified in many ways, including the addition of peptide nucleic acid (PNA) or alternating 2′O-methyl to their backbones, switching ribose rings to morpholine rings (PMO), switching internucleoside bonds with phosphorothioates (PS-ODNs), or by conjugating cationic peptides to PNAs and PMOs[148-150].

Antisense technology using synthetic siRNAs have been used as a powerful tool in knocking down genes in prokaryotes (and also in eukaryotes), including hepatitis G virus[151], *influenza* virus[152], *picornavirus*[153], and *Trypanosoma brucei*[154]. When targeted to essential genes, siRNAs inhibit growth of *E. coli*[148,149,155,156], *S. enterica*[157], *Staphylococcus aureus*[158], *M. smegmatis*[159] and MTB[61,160]. Antisense RNAs have been successfully used for the study of bacterial growth and metabolism since this approach allows conditional knock down of target genes[161-165]. It has also been used for the study of various putative virulence factors in bacteria[166,167]. Antisense technology has helped in identifying new antibiotics[168,169], antibiotic targets[170,171], sensitizing bacteria to antibiotics[171-173], and to elucidate the mechanism of action of potential new drugs[171].

Molecular beacons are a newer class of antisense RNA tagged with a fluorophore/quencher pair and their use for *in vivo* detection and knockdown of mRNA is gaining popularity. Molecular beacon based short interfering RNA (MB siRNA) has recently been proven to be a powerful tool for therapeutic gene silencing because of its specificity, broad applicability, and high efficiency[174-176]. The on/off signals produced by the fluorophore/quencher pair depends on the conformational state of the MB (Figure 7). In the absence of the target mRNA, the stem brings the quencher in close proximity with the fluorophore and turns the fluorescence off with high quenching efficiency *via* fluorescence resonance energy transfer (FRET). In FRET, the energy from the donor chromophore is transferred to acceptor quencher nearby thus resulting in the absence of fluorescence. If the quencher and the fluorophore are far apart (following hybridization of the beacon to its target) then the quencher molecule will not be able to absorb the energy from the donor fluorophore. This would result in an increase in fluorescence. This technology has been used to detect mRNA expression in cells as well as for the detection and knockdown of telomerase expression in human breast cancer cells[177], *BMP4* mRNA in hedgehog signaling[178], aromatase mRNA in breast cancer cells[179], and for the detection and attenuation of *mce4a* mediated *M. smegmatis* infection in macrophages as discussed in section below[155,180].

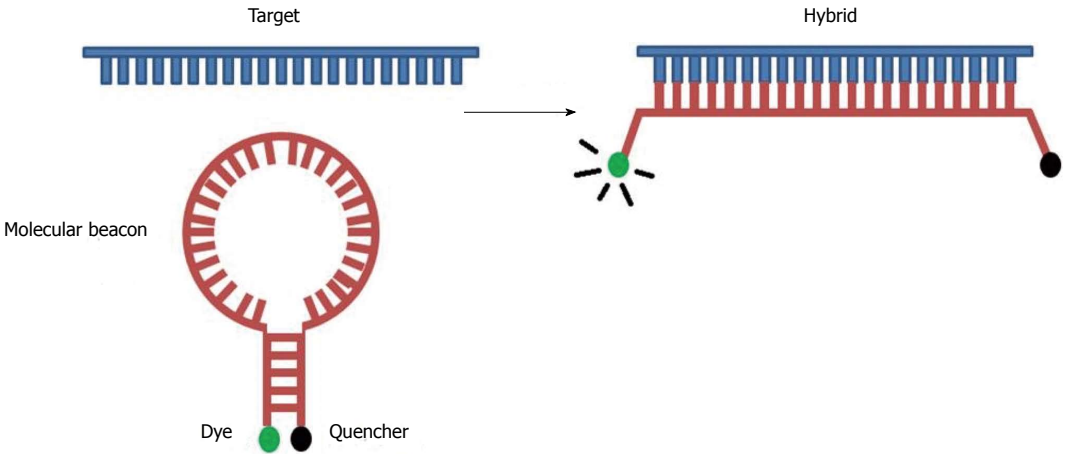


**Figure 7 Molecular beacon technology.** Molecular beacons are hairpin shaped antisense RNAs that does not fluoresce in the absence of mRNA binding (left) while the fluorescence increases when the beacon hybridizes with its target mRNA (right).

## *M. SMEGMATIS* AS AN *IN VITRO* MODEL FOR STUDYING MYCOBACTERIAL INFECTION AND PERSISTENCE

The *mce* operons are widely seen throughout the genus *Mycobacterium* and a homolog of *mce4* has been confirmed in the mycobacterial species *M. smegmatis*[64,181]. Even though *M. smegmatis* is non-pathogenic, previous studies have shown that it can survive and multiply within macrophages in a pathogen-like manner by manipulating the host cell during initial stages by delaying phagosomal acidification and recruitment of V-ATPase[28,182], thus making it a suitable model to study *mce4 operon* mediated invasion and intracellular mycobacterial survival.

## STUDIES IN OUR LAB ON THE ROLE OF MOLECULAR BEACONS IN DETECTING AND ATTENUATING MYCOBACTERIAL INFECTION IN MACROPHAGES

In order to determine the ability of a siRNA molecular beacon to detect and attenuate mycobacterial infection in macrophages, we first conducted experiments towards determining the most infective gene in the mce4 operon. Because of the slow growth rate of MTB and also due to the high degree of homology between *mce4* operons of mycobacteria[181], the *mce4* operon of the rapid growing *M. smegmatis* was selected for our studies. Using gene specific primers with the reverse primer for each set excluding the termination codon, each of the *mce4* genes, *mce4A, mce4B, mce4C, mce4D, and mce4F* were PCR amplified, cloned into the prokaryotic expression vector *pTrcHis2-TOPO* and stably expressed in *E. coli*. Western blot analyses with monoclonoal antibodies against c-myc and 6xHis showed that the MCE4 proteins were expressed in host *E.coli*. Next we conducted invasion assays in MCF7 breast cancer cells using *E. coli* clones expressing the *M. smegmatis* genes and the results showed that *mce4A-F* conferred virulence to its host *E.coli*. However, *mce4A* appeared to confer the earliest virulence to its host *E. coli* and the virulence was found to be sustained during the entire invasion period (72hr)[180]. We later, repeated the cloning experiments using the *mce4* operon genes of MTB, by PCR amplifying each of the *mce4A-F* (Figure 8), cloning into TOP10 *E.coli* using the prokaryotic expression vector *pTrcHis2-TOPO*, and performing invasion assay using MCF7 breast cancer cells. Our results showed that, as with *M. smegmatis*, the *mce4A* gene conferred the greatest degree of virulence to its host *E. coli* (Figure 9). Therefore, *mce4A* was selected as the target gene for designing a molecular beacon antisense RNA.

The *mce4A* antisense molecular beacon RNA was designed to have a stem-loop structure, with the nucleotides in the stem complementary to each other to form a 5-base pair double stranded stem and the loop consisting of 20 nucleotides that are complementary to a region of the target *mce4A* mRNA in *M. smegmatis*. Also, conjugated to the 5' and 3' ends of this molecular beacon are the fluorophore 5' TYE™ 665 and quencher Iowa Black RQ-SP (fluorophore quencher for 500-700 nm spectrum), respectively. This molecular beacon design combined both detection and therapeutic capabilities[177-179]. The rationale is that in the absence of the target *mce4A* mRNA, the molecular beacon remains in its hairpin form while in the presence of its target mRNA the 20 nucleotide loop will compete with the 5 nucleotide stem for hybridization to their target *mce4A mRNA* and the stem to its complementary pairs on the opposite ends of the target sequence. The hybridization potential of the loop to its target, based on the number of nucleotides within it (20 *vs* 5), will be greater than that of the strands for the stem. Hybridization of the loop to the *mce4A* mRNA will separate the fluorophore from the quencher thus inducing fluorescence (detection) and degradation (therapeutic) of the mRNA.

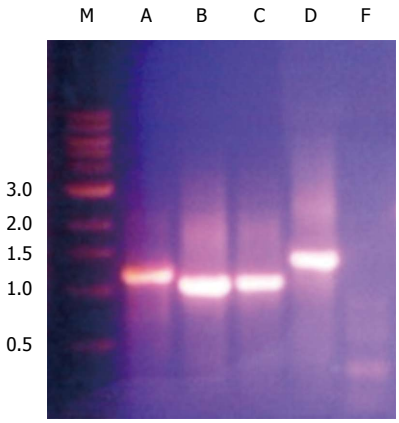


**Figure 8 Polymerase chain reaction amplification of mce4A, mce4B, mce4C, mce4D and mce4F of *Mycobacterium tuberculosis*.** *Mce4* operon genes were polymerase chain reaction amplified from *M. tuberculosis H37Rv* using gene specific primers and resolved on 1% agarose gel. The forward primer spanned the first 21 nucleotides from the beginning of the open reading frame and the reverse primer covered 21 nucleotides spanning the complementary strand to the 3' end of the gene. The termination codon was omitted so that the product, MCE4A-F, will be expressed with a 6XHis tag and a myc tag.


**Figure 9 Mtb-MCE4 proteins confer virulence to *Escherichia coli*.** *Escherichia coli-Mtbmce4* clones were used to infect 2 × $10^6$ MCF7 epithelial cells at an MOI of 10:1 for 2 h. The level of infection was assessed by counting bacterial colony numbers at 24 , 48 and 72 h post-infection ($n$ = 3).

Since the mycobacterium utilizes the product of *mce4A* for entry in to macrophages and for its survival using host cholesterol for carbon and energy source transported through the MCE4 transporters[41,45,62,183], the degradation of the *mce4A* mRNA will lead to its reduced survival. Our studies first tested the ability of the *mce4A* siRNA to detect its target *mce4A* mRNA in *M. smegmatis* and in macrophages infected with *M. smegmatis and* the results show that the molecular beacon siRNA detected its target in *M. smegmatis* and in macrophages infected with *M. smegmatis.* Thus, we were able to show that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* that facilitates the detection of mycobacterial infection in macrophages.

Tests were carried out to test the ability of this siRNA molecular beacon to not only detect but also attenuate mycobacterial infection in macrophages. Towards this end, we used a green fluorescent protein (GFP) expressing lentiviral vector, *piLenti*-siRNA-*GFP,* to successfully transduce and stably express the *mce4A* siRNA molecular beacon construct in macrophages infected with either *E. coli* expressing *mce4A* gene (*E. coli-4A*) or *M. smegmatis.* Using confocal imaging and Western blot analyses with anti-GFP antibodies, we were able to demonstrate stable expression of siRNA up to 48 h post transduction and infection using the GFP reporter.

After confirming the expression of the GFP protein by fluorescence imaging and Western blot analyses, invasion assay was carried out to assess the effect of *mce4A* siRNA on mycobacterial infection in macrophages. For this, differentiated U937 macrophages were transduced with *piLenti-siRNA-GFP* phage for 24hrs followed by infection with *E.coli-4A* or *M. smegmatis* for 3 h, and incubation for 0, 3, 6, 24, and 48 h, respectively. The cells were extensively washed and lysed in 0.1% Triton-X 100 lysis buffer and the lysates were plated on either LB agar containing 100 μg/mL ampicillin for *E. coli-4A* or 7H11 media for *M. smegmatis*. The degree of attenuation of *E. coli-4A* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 0%, 77%, 59.6%, and 99.7%, respectively. The degree of attenuation of *M. smegmatis* infection was compared between 3, 6, 24, and 48 h against that at 0 h baseline and was found to be 94.8%, 70.3%, 98.9%, and 93.4%, respectively. Thus, our results showed that the *mce4A* siRNA was able to significantly attenuate both *E. coli-4A* and *M. smegmatis* infection in macrophages[159].

Separate set of experiments were conducted to further test the hypothesis that the *mce4A* siRNA molecular beacon can attenuate the *mce4A* mRNA levels in *E. coli* expressing *mce4A* gene within infected macrophages. For this, reverse transcription polymerase chain reaction analysis was performed on lysates from differentiated U937 cells which were transduced with the *piLenti-siRNA-GFP* phage for 24 h, followed by infection with *E. coli-4A* for 3 h and incubation for 0, 3, 6, and 24 h. The cells were washed and lysed and the intracellular bacteria were isolated and washed at each time point of incubation. The bacterial sample from each of the time points were lysed and the mRNA was isolated and purified using DNAse 1 enzyme treatment. Reverse transcripts were generated using RTPCR and the cDNAs were amplified using gene specific primers for *M. smegmatis mce4A* and *E. coli 16S* rRNA gene as internal control. The degree of attenuation of *mce4A* mRNA levels was compared between 3, 6, and 24 h against that at 0hr and the results were found to be 0%, 81%, 40%, and 36%, respectively using densitometry gel analysis. Our results thus showed that *mce4A* siRNA was able to attenuate *mce4A* levels within infected

macrophages as opposed to *E. coli 16S* rRNA internal positive control and the degree of attenuation of *mce4A* mRNA levels in *E. coli-4A* was found to be significant.

Thus, we have successfully demonstrated that a molecular beacon can be designed against one of the *mce4* operon genes in *M. smegmatis* which can be used to both detect and attenuate mycobacterial infection in macrophages.

Antisense oligonucleotides, considered the pharmacology of the future[184], interact with their mRNA targets with greater specificity and binding affinity than traditional drugs to their protein targets. Recent advances have enhanced their hybridization to target mRNA, reduced their overall toxicity with decreased susceptibility to cellular nucleases. The lung provides an excellent target for direct antisense oligonucleotide delivery by inhalation, thereby achieving a bolus dose directly to the target site. Cationic lipids in the lung surfactants enhance oligonucleotides entry into cells[185]. Penetration of the inhaled oligonucleotides into deeper tissues of the lung has been established by autoradiogram, surgical dissection and receptor quantification studies[186]. Further studies to test the hypothesis that *mce4* siRNA respirable molecular beacons can localize and attenuate mycobacterial infection in pulmonary granulomas in animal models will take the fight against TB a long way in eradicating this versatile human pathogen.

## CONCLUSION

The association of the *mce* operons, especially that of *mce4*, with mycobacterial invasion and latency is no longer considered casual and with strong evidences emerging over the recent years it can now be considered as a potent mediator of *MTB* infection and survival in its only human host. The mce invasion domain is equipped to mediate the entry and localization of the bacteria in the host macrophages at cholesterol rich regions creating cholesterol-associated protein coated phagosomes, thereby creating an ingenious mechanism for subverting the immune defenses. Another paradigm to the mycobacterial saga was added by the discovery that the MCE associated protein, YRBE4 transporters, in conjunction with the MCE4 domains, transport cholesterol into the cell for its energy and carbon needs, which then possibly generates metabolites that can further mediate its latency in the host. Strategies like identifying the level of infectivity of individual *mce* operon genes and designing efficacious drugs like molecular beacon siRNAs against *mce* targets can aid in the simultaneous detection and eradication of this elusive human pathogen.

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REVIEW

# Computed tomography-based finite element analysis to assess fracture risk and osteoporosis treatment

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## Abstract

Finite element analysis (FEA) is a computer technique of structural stress analysis and developed in engineering mechanics. FEA has developed to investigate structural behavior of human bones over the past 40 years. When the faster computers have acquired, better FEA, using 3-dimensional computed tomography (CT) has been developed. This CT-based finite element analysis (CT/FEA) has provided clinicians with useful data. In this review, the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and effects of osteoporosis medication are overviewed.

**Key words:** Finite element analysis; Bone mechanics; Hip fracture; Osteoporosis; Vertebral fracture; Fracture risk



**Core tip:** Finite element analysis (FEA) is a computer technique of structural stress analysis developed in engineering mechanics. With the faster computers, better FEA, using computed tomography (CT) has been developed. This CT-based finite element analysis (CT/FEA) has provided clinicians with useful data. In this review, the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and osteoporosis treatment are overviewed.

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## INTRODUCTION

Finite element (FE) method is a calculation technique

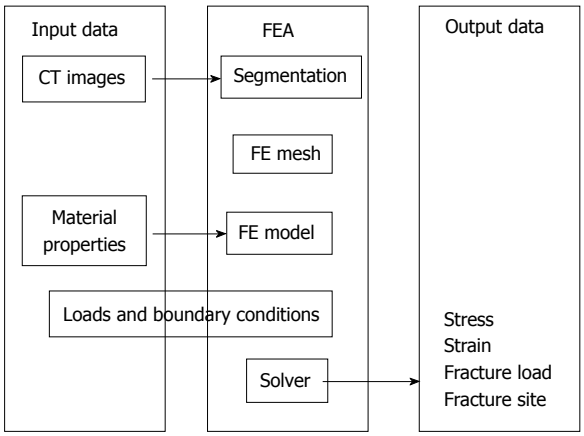


**Figure 1 Steps involved in construction, analysis, and output data of computed tomography-based finite element analysis for human bones.** FEA: Finite element analysis; CT: Computed tomography.

using computers to solve structural mechanics problems. With FE, approximate solutions of boundary value problems are solved. The concept of the FE is the subdivision of the mathematical complex structure into independent components of simple geometry. The number of components is finite, therefore the components are called "finite elements". The response of the mathematical model is considered to be discrete model obtained by connecting or assembling the collection of all elements. When examining artificial and natural systems such as bridge, building, airplane, and skeleton, the disconnection-assembly concept occurs. Calculation of structural mechanics problems using FE consists of dividing the structure into finite elements, and then each element is assigned by the element equations to the problem, followed by recombining all sets of element equations into the whole structure for the final calculation.

Finite element analysis (FEA) is a practical application of FE for performing structural analysis. FEA consists of using mesh generation techniques for dividing a complex structure into small simple elements and using software program with FE algorithm. FEA has been improved over the past 40 years and become an indispensable tool to assess structural mechanics of human tissues such as bones. In 1972, FEA was first applied in orthopedic biomechanics to calculate stress of human bones[1]. Since then, FEA has been widely applied to assess the human bone mechanics[2]. When the faster computers have been available, improved FEA and more advanced imaging modalities have been developed. The FEA using 3-dimensional (3D) computed tomography (CT) data was applied and was used to assess vertebral bone strength clinically in the early 1990s[3]. This so-called "computed tomography-based finite element analysis (CT/FEA)" has provided clinicians with useful data. Clinical applicability of CT/FEA of human bones might depend on accuracy, reliability, the ease of use, and usefulness. The purpose of this review article is to overview the mechanism of CT/FEA, validation studies of CT/FEA to evaluate accuracy and reliability in human bones, and clinical application studies to assess fracture risk and osteoporosis treatment.

## MECHANISM OF CT/FEA FOR HUMAN BONES

There are three major stages, *i.e.*, pre-process, solution, and post-process, in CT/FEA for human bones[4]. The stages are summarized in Figure 1. The FE model is generated in the first pre-process stage and the problem of the model is solved by FE solver in the second solution stage. The solved results are analyzed in the post-process stage. The first step of CT/FEA is to acquire CT images of human bones, and then transfer the data to FE modeling system. After CT data are acquired, the region of interest (ROI) is segmented and mesh generation is proceeded. The quality and accuracy of the FE model is highly dependent on the ROI segmentation and mesh generation. A 3D image constructed with CT scanning is called voxel. In order to create FE model accurately and good quality, it is essential to define the anatomical bone shapes and segment the ROI accurately. After the ROI is segmented and the 3D bone geometry is obtained, mesh generation procedure can be done. There are several mesh generation techniques. In voxel based mesh generation technique, a pre-defined voxels from the CT scanning constitutes elements. In voxel based mesh, there is no surface or solid bodies. On the other hand, geometrical model with surface points is generated in structure based mesh procedure. Structure based mesh can generate more complex mesh and shell-solid mesh. In shell-solid mesh, component is modeled with two types of elements. Trabecular bone is modeled using solid elements. Outer surface of the trabecular bone, cortical bone or cortical shell is modeled using thin plate shell elements.

After FE model is generated, material properties of the each element in the model is assigned. The density of the each element is defined using the correlations between the Hounsfield unit (HU) value of the CT and the apparent density. The Young's modulus of the element is defined using the equations between the density and the mechanical properties of the bone. Regarding FE modeling of the human bone, there are two problems: (1) material properties of the cortical bone and the trabecular bone are very different; and (2) the material properties distribution within the cortical bone and the trabecular bone is not homogeneous. One of the methods to deal with the first problem is to use shell-solid mesh and model the cortical bone and trabecular bone using two types of elements. Cortical bone is constructed with "shell" elements. Trabecular bone is modeled using "solid" elements. It is essential to assure that assigned bone stiffness is connected to the assigned bone mineral density (BMD). The HU value of a voxel is connected to the bone stiffness. The HU values of a CT image are applied to define the

appropriate bone stiffness in the FE model.

An essential procedure to define material properties in FE model consists of calibrating the CT density, relating the voxel value to BMD, and accurately defining Young's modulus of the bone. For this purpose, a calibration phantom, which consists of known concentration of calcium hydroxyapatite, is used in CT image acquisition. By normalizing the HU values with the known concentration of the calibration phantom, an equation between CT density and BMD of the bone is established. It is shown that the apparent density and the mechanical properties of the bones are related with equations using power laws[5].

It is preferable that trabecular bone shows anisotropic mechanical behavior. Therefore, CT/FEA with anisotropic properties of the bones is assumed to be more accurate. In some studies, no significant difference was shown between the results by isotropic CT/FEA models and by CT/FEA orthotropic models[6]. But another study indicated a significant difference between the isotropic CT/FEA models and orthotropic CT/FEA models[7].

The final step is to apply the loads and boundary conditions in FE model. Boundary conditions defines that assigned nodes of the elements are limited in their degrees of freedom and are constrained. The constrained nodes are fixed or prescribed to move in a fixed amount. The applied loads work for deforming the element and producing internal stress. The solver works to solve the equilibrium between applied loads and internal material stress under the loads and boundary conditions. The location of the fixed element is defined by constraining the assigned nodes of the element completely. It is essential to apply the accurate loads and boundary conditions to fairly assess the mechanical behaviors in bone in daily activities.

## CT/FEA SOLUTION AND CLINICAL APPLICATION

In order to apply CT/FEA in human bones, two important characteristics of bone have to be considered. First, bone is failed because of overstress or insufficient bone strength. Second, bone is adapting and remodeling by the applied loads and stresses, known as Wolff's law. Because of this bone tissue adaptation process, the mechanical properties of bone are changed.

In our increasingly aging societies, there are more patients with osteoporosis. Osteoporotic patients have much higher bone fracture risk due to insufficient bone strength. Fracture often occurs at hip, spine, humerus, and forearm. To determine whether pharmacological intervention for osteoporosis is needed, accurate fracture risk assessment is helpful. Traditionally, measurement of BMD by dual energy X-ray absorptiometry (DXA) has been the standard method for diagnosing osteoporosis. However, BMD itself is not able to predict the actual fracture in a reliable way. It has been reported that many patients which have normal BMD developed fractures due to osteoporosis[8]. The correlation between BMD and experimental fracture load was reported 45%-57%[9,10]. Recently, fracture risk assessment tool (FRAX) was developed to assist with clinical treatment decisions. FRAX was developed by the World Health Organization Collaborating Center for Metabolic Bone Diseases and calculates the 10-year probability of major osteoporosis related fractures (hip, spine, humerus, and forearm)[11-13]. FRAX can only be used in untreated patients. FRAX is useful for primary screening for osteoporosis and related fractures, but not useful for assessing therapeutic effects.

In CT/FEA, the stress and strain distributions are able to be assessed. Stress, strain, fracture load, and fracture site are assessed as a post-processing stage. Fracture is defined, and fracture load and fracture site are predicted with fracture criterion. For assessing the failure of engineering and biological materials, stress-based criteria or strain-based criteria have been used. Fracture criteria of bone include von Mises stress[14-16], Drucker-Prager stress[16-18], maximum principal strain[19], maximum principal stress[19], and minimum principal strain[17]. Some studies reported that bone fracture is regulated by strain and stress[20,21].

## VALIDATION OF CT/FEA

After FE model with material properties is constructed, loads and boundary conditions are assigned, and CT/FEA solver solved the model simulation, the results are transformed into a useful output data, *i.e.*, stress, strain, fracture load, fracture site, to meet the investigation purpose. In addition, accuracy and validity of the CT/FEA should be shown through validation of the model. By comparing CT/FEA output data with the measured values in mechanical testing, CT/FEA can be validated and verified. It has been impossible to measure strains or stresses inside the trabecular bone structure with conventional methods. Therefore, strains at the bone surface are experimentally assessed, and it is considered that a validation of the surface strains substitutes for validation of the strains and stresses inside the bone. Strain gauges can measure strains at the site it attached. With strain gauges, strains at the some parts of specimen surface can be measured through mechanical testing. Recently, full-field strain measurement techniques that can measure surface strains throughout the specimen have been improved. Digital image correlation technique has been used for the measurement of surface strains during mechanical testing[22-24]. Digital image correlation has been successfully used for validation of the CT/FEA of the proximal femur[25].

For clinicians, fracture load, fracture pattern, and fracture site are the most useful data. There are some *ex-vivo* validation studies of CT/FEA analyzing the fracture load and then evaluate the accuracy by performing mechanical testing with human cadaveric

**Table 1 Validation studies of the proximal femur**

| Ref. | Software | Variable | $R^2$ | Slope |
|---|---|---|---|---|
| Cody *et al*[9] | EBE-PCG | One-legged stance fracture load | 0.84 | 0.85 |
| Keyak *et al*[14] | ABAQUS | Stance configuration fracture load | 0.75 | 0.99 |
| Keyak *et al*[14] | ABAQUS | Fall configuration fracture load | 0.90 | 1.24 |
| Keyak[27] | ABAQUS | Stance configuration fracture load | 0.93 | 1.15 |
| Bessho *et al*[18] | Mechanical Finder | Stance configuration fracture load | 0.96 | 0.94 |
| Bessho *et al*[18] | Mechanical Finder | Principal strain | 0.93 | 0.91 |
| Tanck *et al*[15] | MARC | Stance configuration fracture load | 0.92 | 1.07 |
| Derikx *et al*[16] | MSC (von-Mises criterion) | Stance configuration failure force | 0.91 | 0.92 |
| Derikx *et al*[16] | MSC (Drucker-Prager criterion) | Stance configuration failure force | 0.91-0.94 | 0.93-1.01 |
| Dragomir-Daescu *et al*[28] | ANSYS Mechanical APDL | Sideways fall fracture load | 0.86 | 1.36 |

Used software of finite element analysis, validation variables, cross-validation $R^2$ values, and slope of the regression line are summarized.

**Table 2 Validation studies of the spine**

| Ref. | Software | Variable | $R^2$ | Slope |
|---|---|---|---|---|
| Silva *et al*[29] | ABAQUS | 10 mm thick vertebral section yield load | 0.91 | 0.86 |
| Martin *et al*[30] | ADINA | Failure load | 0.79 | 0.57 |
| Crawford *et al*[31] | ABAQUS | Compressive strength | 0.86 | 0.72 |
| Imai *et al*[17] | Mechanical Finder | Failure load | 0.96 | 0.88 |
| Imai *et al*[17] | Mechanical Finder | Minimum principal strain | 0.70 | 0.93 |
| Kinzl *et al*[33] | ABAQUS | Apparent strength | 0.92 | 1.02 |

specimens. For hip, validation studies indicated that CT/FEA might be able to predict femoral strength more than quantitative CT (QCT) or DXA[9] and was able to assess fracture site of the proximal femur[26]. Nonlinear CT/FEA showed more accurate predictions of fracture load at proximal femur[27]. Using shell-solid meshing, nonlinear CT/FEA analyzed fracture loads of the proximal femurs as well as principal strains of the surface of the femoral bones accurately[18]. Using Drucker-Prager yield criterion, CT/FEA could predict femoral bone strength and fracture site better than using von Mises yield criterion[16]. Validation studies of simulating a sideways fall on the hip revealed that CT/FEA predicted fracture loads and fracture patterns with a high degree of accuracy[28]. The validation studies of the proximal femur are summarized in Table 1.

Regarding spine, the cadaver studies have validated and verified that CT/FEA predicts failure loads and fracture patterns for vertebral sections with 10 mm thickness[29]. *Ex-vivo* validation studies of CT/FEA analyzing vertebral bones demonstrated that CT/FEA could predict vertebral compressive strength more accurate than BMD[30] and QCT[31]. Nonlinear CT/FEA using shell-solid meshing and Drucker-Prager yield criterion was able to predict distribution of minimum principal strains of the vertebra, vertebral strength, fracture pattern, and fracture site accurately[17,32]. Nonlinear FEA using high-resolution peripheral quantitative CT could predict vertebral strength well and the analyzed pressure distributions were qualitative agreement with the experiments measured by pressure sensitive films[33]. The validation studies of the spine are summarized in Table 2.

## ASSESSMENT OF FRACTURE RISK AND OSTEOPOROSIS TREATMENT USING CT/FEA

Based on validation and verification with the cadaver studies, CT/FEA has been applied clinically in the assessment of fracture risk and osteoporosis treatment. In clinical data, the hip fracture risk index in discriminating hip fracture derived from CT/FEA was significantly better than total hip BMD and DXA-based structural engineering models[34]. The strength of the proximal femur varies depending on the specific force configuration.

In fall configuration, the force magnitudes and directions can be influenced by many biomechanical factors such as body weight, height, and position. The study to assess the relationship between femoral bone strength by nonlinear CT/FEA and incident hip fracture in multiple loading conditions, posterolateral loading in men and posterior loading in women were most strongly associated with incident hip fracture[35].

As for vertebral fracture, nonlinear CT/FEA had higher discriminatory power for vertebral fracture than lumbar spine BMD by DXA and volumetric BMD by QCT[36,37]. Consequently, CT/FEA has the potential to replace DXA and QCT in discriminating osteoporosis related fractures.

Regarding osteoporosis treatment, CT/FEA was useful for assessing teriparatide and alendronate medication effects at the lumbar spine[38]. In addition, a study using nonlinear CT/FEA clinically showed that vertebral compressive strength by CT/FEA was a significantly

better predictor for vertebral fracture than BMD, and was able to assess medication effects significantly earlier than BMD[36,37].

## CONCLUSION

Osteoporosis related hip fractures and vertebral fractures have become a major social problem because the elderly population continues to increase. It is essential to assess fracture risk, start medication, and prevent fractures in the management and treatment of osteoporosis. In this article, the mechanism of CT/FEA, validation studies of CT/FEA for human bones, and clinical application studies to assess fracture risk and osteoporosis treatment are reviewed. CT/FEA accurately assesses bone strength and fracture site and is useful for assessing fracture risk and medication effects on osteoporosis. CT/FEA also assesses bone strength under various loading configurations normally seen in daily living activities[39]. With CT/FEA in the diagnosis and management of osteoporosis, patients and their clinicians are able to tailor a treatment plan according to a patient's specific clinical scenario.

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MINIREVIEWS

# Multiplex planar microarrays for disease prognosis, diagnosis and theranosis

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## Abstract

Advanced diagnostic methods and algorithms for immune disorders provide qualitative and quantitative multiplex measurement for pre-clinical prognostic and clinical diagnostic biomarkers specific for diseases. Choice of therapy is confirmed by modulating diagnostic efficacy of companion, theranotic drug concentrations. Assay methods identify, monitor and manage autoimmune diseases, or risk thereof, in subjects who have, or who are related to individuals with autoimmune disease. These same diagnostic protocols also integrate qualitative and quantitative assay test protocol designs for responder patient assessment, risk analysis and management of disease when integrating multiplex planar microarray diagnostic tests, patient theranostic companion diagnostic methods and test panels for simultaneous assessment and management of dysimmune and inflammatory disorders, autoimmunity, allergy and cancer. Proprietary assay methods are provided to identify, monitor and manage dysimmune conditions, or risk thereof, in subjects with pathological alterations in the immune system, or who are related to individuals with these conditions. The protocols can be used for confirmatory testing of subjects who exhibit symptoms of dysimmunity, as well as subjects who are apparently healthy and do not exhibit symptoms of altered immune function. The protocols also provide for methods of determining whether a subject has, is at risk for, or is a candidate for disease therapy, guided by companion diagnosis and immunosuppressive therapy, as well as therapeutic drug monitoring and theranostic testing of disease biomarkers in response to immunoabsorption therapy. The multiplex test panels provide the components that are integral for performing the methods to recognized clinical standards.

**Key words:** Simultaneous methods; Multiplex planar microarrays; Disease integrated panels; Theranosis; Prognosis; Diagnosis



**Core tip:** Multiplex planar microarrays integrated for simultaneous, quantitative methods of prognosis, diagnosis, and theranosis provide a powerful technology for comparative measurements of changes in disease states and risk analysis, especially for autoimmune diseases.

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diagnosis and theranosis. *World J Exp Med* 2015; 5(3): 188-193 Available from: URL: http://www.wjgnet.com/2220-315X/full/v5/i3/188.htm DOI: http://dx.doi.org/10.5493/wjem.v5.i3.188

## INTRODUCTION

Recent investigation of genomic and proteomic technologies have provided data for pathological conditions of immune function. Studies of human body fluids have confirmed that antigens, their specific antibodies as well as inflammatory mediators, to be diagnostic markers specific for inflammatory, allergic and autoimmune diseases. These proteomic studies have diagnostic and therapeutic benefits[1]. Serum auto-antibodies and inflammatory indicators are detectable even before onset of clinical symptoms and also during the course of systemic and organ-specific dysimmunity. Immune serum-derived markers become predictive biomarkers of disease in healthy subjects and markers of disease activity and severity in patients. New multiplex diagnostic technologies being introduced in laboratory medicine[2] allow the simultaneous detection of several different auto-antibodies and biomarker analytes for screening purposes in high-risk groups. Auto-antibodies with demonstrated diagnostic and predictive roles in organ-specific systemic disease are reviewed[3]. Cohort studies have shown that patients may have carried auto-antibodies and other markers of dysimmunity for extended periods of time, not diagnosed until discovery of other related clinical symptoms, as reported by Bizarro[4]. Serum auto-antibody assays have clinical utility in autoimmune diseases, including Addison's disease, celiac disease, Crohn's disease, biliary cirrhosis, Hashimoto's thyroiditis and type-1 diabetes. Dysimmunity is genetically and immunologically complex. Early diagnostic biomarker discovery is becoming increasingly more important for early diagnosis and early treatment in these diseases. Proteomic analysis of body fluids provides a non-invasive methodology for early diagnosis in many different disease settings. Proteomics-based approaches have made steady progress into biomarker discovery and understanding of autoimmune, allergic and cancerous diseases[5]. Traditional diagnosis, diagnostic proteomic methods including targeted antibody-based protein arrays, which have been particularly informative in the field of autoimmunity, are compared by Wu and Mohan[6].

Auto-antibodies are specific markers of autoimmune disease and humoral autoimmunity. Fluctuations in acute phase reactants, complement, cytokines, chemokines and growth factors suggest systemic and cell-mediated dysimmunity. Clinical understanding of autoimmune and allergic diseases includes analysis of auto and allo-antibody class IgG, IgM, IgA and IgE immuno-globulins. Multiplex, simultaneous identification measures different auto-antibodies in sera of patients suffering from autoimmune diseases.

The simultaneous measure of correlating analytes in reduced test volumes of patient's samples and test reagents, plus faster diagnosis, lowers assay costs and provides more diagnostic results in comprehensive serological profiles. Improved quantitative data is obtained by high-throughput techniques such as planar multiplex microarrays for antibody and analyte diagnostic profiling[7]. Recently collected data demonstrate more accurate analytical sensitivity and reproducibility. Aspects of multiplex assay sensitivity, specificity, co-efficient of variation and data interpretation are becoming standards of these new and promising clinical applications[8].

Gibson *et al*[9] present results to demonstrate that discrete, individual, clinical, laboratory or radiological parameters are limited to accurately diagnose or predict disease outcomes. Biomarker protocol panels which diagnose at an earlier time point, indicate early diagnosis to guide therapeutic strategies for dysimmune conditions towards more effective clinical management. There is a growing need for deeper understanding of multi-factorial immune disorders. Proteomic platforms offering a multiplex approach are more likely to reflect the complexity of dysimmune disease processes. Diagnomic approaches aid in early detection and are beneficial in guiding immune disorder treatment towards earlier disease prevention. Integrated panels facilitate these aims by offering comprehensive etio-pathogenesis test panels to screen asymptomatic and diagnostic symptomatic subjects, for a multitude of autoimmune, dysimmune and allergic conditions early in their disease course to monitor disease activity and severity for more effective treatment.

## ANALYTICAL ATTRIBUTES

Autoimmune diseases include twelve currently recognized systemic, sixteen systemic vasculitis, eight idiopathic inflammatory myopathies, four immune-mediated systemic, thirteen endocrine and reproductive, five hepatobilliary and pancreatic, four gastrointestinal, four cutaneous, six cardiovascular and pulmonary, eleven neurological, four occular, five renal and eleven hematologic autoimmune diseases to be active in serious illnesses found in almost every human organ system[10]. The increasing incidence of autoimmune pathologies, *e.g.* rheumatoid arthritis (RA) in aging populations, is reported in the scientific literature by Lea *et al*[2]. Allergies are increasing in prevalence, impacting the burden of health care costs. Allergic rhinitis, asthma, atopic eczema and food allergy are known as co-causes of chronic ill health[11].

Antibodies to endogenous self-antigens or foreign allergens continue to be detected and recognized as reliable disease biomarkers for autoimmune and IgE - mediated allergic diseases. For example[2], a patient may have RA without experiencing symptomatic pain, which

can delay early diagnosis. Typically, autoimmune diseases develop slowly. In RA. Early prophylactic detection leads to early treatment at efficacious drug concentrations, with potential for differential diagnostic pre-symptomatic recovery, especially in patients who respond to drug therapy and/or in whom immunosuppressive treatment would be of benefit. The treatment method of choice may include specific therapeutic antibodies which differ from standard autoimmune treatment. New and more specific biomarkers indicative of various dysimmune pathologies continue to be recognized and accepted as providing disease confirmation, facilitating differential diagnosis, disease activity monitoring and developing therapy guided by companion diagnostics. This key attribute relates to companion diagnostics, wherein molecular assays that measure levels of proteins, genes or specific mutations provide a specific therapy by stratifying disease status, selecting the proper medication and tailoring dosages to the patient's specific needs. As a result of more accurate proteomic assessment, the FDA (United States) continues to set more demanding guidelines. Therapeutic products require validation based on diagnostic test to conform to label, safety and effectiveness claims. The increasing need for companion or personalized tests is demanded by subsets of treated, positive responders to possible side effects.

Detection of single, discrete biomarkers in isolated test outcomes results in a number of patients as misdiagnosed for autoimmune disease. Incorrect test outcomes are directly caused by patient samples that may contain unstable biomarker antigens, auto-antibodies, endogenous antibodies, patient sample proteins and antibody-antibody reactions. These analytes are often unstable or interfered with in patient's test samples as a direct result of time dependent storage leading to breakdown and cross-contamination, including effects of proteolysis, oxidation and protein interaction. Repeat testing of such stored samples confounds result of confirmatory tests. New diagnostic methods and algorithms for immune disorders need to provide robust, simultaneous and comprehensive, qualitative and quantitative multiplex measurement for prognostic and diagnostic biomarkers specific for autoimmune diseases, while modulating companion diagnostic efficacy of theranostic drug concentrations. Assay methods help to identify, monitor and manage autoimmune disease or risk thereof, in subjects who have an autoimmune disease, or are cohort related. Therefore, it is a benefit to provide integrated indicator measurement using multiplex planar microarray diagnostic tests, including test panels designed for responder patient theranosis assessment, risk analysis and management of disease.

## DIAGNOSIS MANAGEMENT AND THERAPY

The panels provide for diagnosis, management and therapy of immune disorders and dysimmunity. In particular, they provide diagnostic tests, test panel design, methods of use and kits for simultaneous integration of qualitative and quantitative multiplex planar microarray prognostic and diagnostic tests, therapeutic drug concentration tests, drug efficacy tests, companion diagnostics tests and methods of use applied in specific test panels for simultaneous assessment, determination of risk and management of immune disorders. Specific disease panels include tests for genetic predisposition and also measure biomarker responses to treatment efficacy, such as drug dose concentration and drug pharmaceutical efficacy. This data will determine the optimal drug dosage for each patient as the drug level is maintained at the pharmaceutical efficacy level for that patient. Test panels are also designed to facilitate earlier detection of multifactorial etiologies, differential diagnosis from related disease phenotypes and detect disease activity indices such as remission, flare-up, relapse and life-threatening organ involvement. In assessing the risk of whether a patient has or, at some point in the future is prone to develop an autoimmune disease, wherein the method includes: (1) obtaining a sample from the subject; (2) multiplex array measurement of inflammatory indicators, antibodies of various classes to a plurality of different endogenous antigens or allergens in the patient's sample; (3) comparing the same analytes simultaneously measured in reference subjects, or to previous blood draws from the same patient, and or comparing reference patients having a similar immune disorder; and (4) identifying the risk that the patient has, or might develop an dysimmunity based on the comparison in step (3) above. Risk is present when levels of one or more of inflammatory mediators or antibodies to the different antigens or allergens are elevated in the subject's sample as compared to samples from reference patients with clinically normal functions, and/or are elevated in, or at about the same level in the subject's sample.

The patients samples as contemplated in the above method, can be biological body fluids, including peripheral blood, serum, plasma, cerebrospinal fluid, synovial fluid, bone marrow, saliva and urine samples.

In another method to qualify immune disorder patients, as eligible to receive disease-modifying therapy, the method comprises: (1) obtaining a sample from the immune disorder patient; (2) measuring the levels of each of a plurality of different analytes in the patient's sample; (3) comparing the levels with the level of the same analyte measured in samples from reference subjects with clinically normal function, or from reference subjects having disease corresponding immune disorder; and (4) identifying the immune disorder patient as eligible to receive disease-modifying therapy based on the comparison in step (3).

In another embodiment, the analysis relates to a method for identifying and selecting a patient with immune disorder for therapeutic drug monitoring, wherein the method comprises: (1) confirming clinical decision

making and diagnostic algorithms so that multiplex planar diagnomic microarray tests and disease panels can be implemented to monitor treatment efficacy; (2) test panels are integrated for simultaneous, qualitative and/or quantitative prognosis, diagnosis and theranosis that may include measurement of drug concentrations and efficacy; (3) test panels allow patients and physicians to make scientifically based medical, individualized decisions about their disease and its therapy; (4) theranostic panels are defined as biomarker tests that specify the dose and efficacy of therapeutics in reference to a patient's genotype, disease phenotype and serological profile, in conjunction with bioinformatics technologies that analyze and interpret human disease and drug response interaction; (5) antibody and biomarker levels are compared as indicators of disease predisposition, stage of development, aggressiveness, drug efficacy, dosing and toxicity; and (6) test panels form a core component of a new personal medicine to transform clinical practice into definitive, diagnostic science.

Disease test panels incorporating biomarkers that confirm pre-symptomatic disease indicators allow targeted disease therapy. Disease stratification lies in correlation of molecular heterogeneity of disease and heterogeneity of response to therapy. The targeted drug must be present and act on maintaining or deteriorating disease symptoms of the patient state to show efficacy. This target would therefore identify a biomarker to determine whether the patient is a likely responder for treatment with a specific therapy. For example, a multiplex planar microarray fluorescent immunoassay, provides quantitative measurement for anti-TNF drug (Tumor Necrosis Factor) concentration for Infliximab, which is used to treat patients with Crohn's disease and RA[2]. Infliximab Research Use Only assays provide 100% specificity. Results of this testing correctly reported the absence of the drug in healthy serum samples, but accurately reported drug levels when expected to be present, for example in RA patients responding to treatment. This novel microarray assay system detects and measures TNFu blocker drug concentrations at high sensitivity and specificity. Measuring the level of the TNF-alpha for anti-TNF-$\alpha$ therapy also provides evidence of treatment efficacy. Additional markers for [rheumatoid (RA)] disease activity, when measured simultaneously with anti-TNF-alpha drug and TNF-units would indicate disease remission.

Biomarker protocol panels identify patients responding to specific therapeutics, as disease progression and treatment efficacy is monitored. Simultaneous measurement of several disease parameters improves benefits of treatment for a patient. These biomarker panels identify the most effective drug for a specific patient. Retrospective drug efficacy analysis following clinical trial with subgroup analysis, clarifies response variations.

Diagnostics are rapidly developing for the management of diseases and emergence of personalized medicine and drugs. As the uniqueness of each individual disease process is confirmed, so is a growing assurance that particular drugs work better in subsets of patients that could not previously he clearly identified or segregated.

Clinical trials have confirmed that subsets of patients may derive no benefit or were harmed by the drug being investigated. A primary benefit for improved, marker-driven patient selection is to determine who is most likely to benefit from a proposed treatment and to exclude patients likely to be harmed by a drug. Advanced protocol test panels efficiently combine development of drugs with companion diagnostic tests.

Accordingly, multiplex analyses are combined in test protocol formats to detect one or more indicators for refining diagnosis and patient selection for treatments. Panels of biomarkers need to be monitored during clinical trials to confirm effective therapy, while simultaneously testing for and confirming drug side effects.

Biomarker based test panels have utility in all phases of clinical work to better understand and confirm disease onset, progression, symptoms and mechanisms of drug action in a population prior to drug approval by regulatory bodies.

Validation for disease status prediction, or dysimmunity, results from clinical accuracy, reproducibility, predictive value and diagnostic efficiency in detecting disease. The challenge in disease validation is variation of biomarker response observed in patient populations. As newly validated biomarkers transition to clinical diagnosis, it should progress through analytical validation including clinical performance and specifications. Clinical validation includes recognition of early stage disease, disorders and clinical parameters to clearly differentiate from similar disease phenotypes. Assays are then tested systematically to standard, reproducible, high-throughput formats.

Autoantibodies to endogenous autoimmune antigens can be identified in apparently healthy individuals. As components of the idiotypic network, these immune-globulins may multiply to cause patho-physiological impact[12]. Such auto-antibodies against endogenous autoimmune antigens can be monitored using multiplex diagnostic tests, methods and kits. The quantitative measurements of these auto-antibodies and other inflammatory mediators clinically evaluate subjects at risk of developing or currently suffering from autoimmune disease or other immune disorders and also be used to monitor efficacy of a treatment regimen. Particularly advantageous are confirmatory testing of patients who exhibit immune disorder symptoms, screening tests for apparently healthy patients with no symptoms of dysimmunity, who are likely to have symptoms in due course.

## DIAGNOSTIC TEST METHODS

Integrated diagnostic test panels provide for assessment, risk analysis and management of immune disorders. In one embodiment, the panels provide means for risk stratification when developing an immune

disorder and in assessing the response to treatment. The methods entail protocols for assaying samples from the subject for panels of analytes, wherein the presence of elevated levels of one or more of the different analytes indicates the presence or risks of an immune disorder. These methods incorporating multiplex planar microarray diagnomic panels, integrate for methods of prognosis, diagnosis and theranostic companion diagnostics. Physical examination, medical history and histopathology, completes a differential diagnosis for autoimmune disease. Tests can be carried out on asymptomatic patients or patients having risk factors or symptoms of the disease. When screening and a patient tests positive for elevated levels of one set of analytes, the patient is assessed for one or more additional confirmatory tests.

A method of assessing risk of vascular pathology includes assaying a sample for auto-antigen and for auto-antibody specific for that antigen. A presence of auto-antigen or auto-antigen reactive to-antibody, indicates risk of autoimmune disease. Increasing levels of auto-antigen and auto-antibodies to autoimmune disease antigens elevates risk for autoimmune disease. In one embodiment, the analytes being assayed together with auto-antibodies, may contain as part of a test panel, biomarkers that are not specific to a particular autoimmune disease, but indicate disease activity and a generalized dysimmune or inflammatory condition. Moreover, at some stages of inflammation and dysimmunity, the tested analyte level may decrease rather than increase over time, to provide valuable diagnostic or prognostic information for evaluating risk of autoimmune disease for endogenous disease specific analytes, for a specific immune disorder or dysimmune condition.

## THERAPY SELECTION

Selected test panels determine whether a patient at risk or diagnosed with an immune disorder, is a candidate for immuno-suppressive or immuno-absorption therapy. The method assays a sample for a plurality of analytes, wherein the presence of altered levels of at least one of the analytes or biomarkers indicates that dysimmunity may be contributing to the subject's immune or inflammatory disorder and the risks thereof. Therapy can be applied together with one or more other tests, physical examination and taking of medical history in accordance with standard practice for differential diagnosis of immune or inflammatory disorders.

Approving a patient with immune disorder to receive therapeutic immune-modulation considers measured levels of serum autoantibodies or inflammatory mediators using a panel protocol of different endogenous analytes, in comparison to known reference samples with clinically normal function and confirmed reference autoimmune disease. A patient would receive immunotherapy if levels of at least one analyte are higher or lower than normal. A patient with an elevated level of at least one analyte may also be treated with immune-suppressive therapy or immune-absorption therapy in accordance with standard practice.

## MULTIPLEX PLANAR MICROARRAY PANEL ASSAYS

Multiplex planar microarray disease assay panel markers, when printed into a microarray multiplex planar assay promote the integration of differential diagnosis, evaluating disease severity and duration, disease prognosis and monitoring response to treatment including therapeutic monoclonal antibodies, on an individual patient basis[13]. Each panel is comprised of biomarkers and therapeutic biologicals that are used in the diagnosis, differential diagnosis, prognosis and theranostic monitoring of a patient in a general disease classification, *e.g.,* autoimmune diseases. Some analytes are part of the clinical criteria for defining a particular autoimmune disease such as Rheumatoid Factor in RA. Additionally, several phenotypes of other autoimmune disease may be co-existant, confounding a diagnosis based on clinical grounds alone. For example, arthritic disease includes RA, idiopathic arthritis, polyarthritis, osteoarthritis and mixed phenotypes (psoriatic arthropathy, arthritis with inflammatory bowel disease). Each may have particular markers and associated treatments. Their inclusion into one panel aids in the differential diagnosis (Bayes Theorem; decision tree analysis) in terms of positive predictive values and odds ratios for ruling in or out a particular phenotype based on clinical presentation and results from laboratory testing.

Prognostic markers to ascertain the disease duration, severity, activity (remission or relapse), organ system involvement and response to effective treatment are also included in the multiplex planar microarray disease panel assays. Where applicable, measurable biological drugs, especially therapeutic monoclonal antibodies, also constitute theranostic data to monitor how diagnostic markers fluctuate in response to treatment and to measure pharmacokinetics of the biologics.

Diagnomic marker analyte classes include autoantibodies, serum immune-globulins, immune complexes, therapeutic antibodies detected as IgG, IgM or IgA immunoglobulin classes, as well as antigen capture formats for acute phase reactants, cytokines, chemokines, growth factors and inflammatory mediators. Panels for Autoimmune Disease include Arthritis, Vasculitis, Nephritis Hematologic Disorders, Celiac and IBD and Thyroiditis.

## CONCLUSION

These multiplex planar microarray disease panel quantitative assays diagnose particular autoimmune disease, such as RA, vasculitis, nephritis, celiac disease and

Crohns' disease. Similar panels can be designed for other inflammatory conditions and allergic diseases. Multiplex Planar Microarray Assays can also be used for differential diagnosis of a particular disease subtype in a spectrum of related diseases as in RA *vs* osteoarthritis or juvenile idiopathic arthritis. Disease prognosis in specific disease or spectrum of related diseases, with detection of disease activity, severity and organ system involvement markers, is an additional benefit. Multiplex Planar Microarray Assays can be applied to pharmacokinetic studies and therapeutic drug monitoring of biological drugs used to treat cancer and autoimmune diseases (*e.g.,* therapeutic monoclonal antibodies), as well as biomarkers that fluctuate in response to treatment.

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ORIGINAL ARTICLE

Retrospective Study

# Eribulin for heavily pre-treated metastatic breast cancer patients

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Author contributions: Digklia A collected data; Voutsadakis IA conceived and designed the project, analyzed data and wrote the paper.

Institutional review board statement: As per our institution guidelines, no IRB approval is needed for retrospective reviews of patients whose care has directly involved or been supervised by an investigator.

Informed consent statement: Given that the study was retrospective in nature and the fact that treatments had been provided according to standards of care, no specific informed consents were needed or obtained from individual patients. Anonymity was guaranteed.

Conflict-of-interest statement: We have no conflicts of interest regarding this study.

Data sharing statement: No additional data are available.



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## Abstract

**AIM:** To discuss treatment with eribulin in clinical practice outside a clinical trial.

**METHODS:** Archives of patients treated for metastatic breast cancer were reviewed and 21 patients treated with the new chemotherapeutic eribulin mesylate, a synthetic analog of a natural marine product, were identified. Information on patients' characteristics and treatment outcomes was extracted. Treatment with eribulin mesylate was initiated at the recommended dose of 1.4 mg/m$^2$ on days 1 and 8 of a 21-day cycle in 17 patients and at a decreased dose of 1.1 mg/m$^2$ on days 1 and 8 of a 21-day cycle in 4 patients due to comorbidities and frailty. Efficacy of the drug was evaluated using the revised Response Evaluation Criteria in Solid Tumors criteria. Progression-Free Survival and overall survival (OS) were calculated using the Kaplan-Meier method starting from the date of eribulin therapy initiation to the date of disease progression documentation or death, respectively.

**RESULTS:** The median age of patients at the time of eribulin mesylate treatment was 53 years (range 34-75). Sixteen patients had estrogen receptor (ER) and/or partial response (PR) positive disease and 5 had ER/PR negative disease (all triple negative). Eight patients had received 2 or 3 previous lines of chemotherapy

for metastatic disease and 13 patients had received 4 or more lines of treatment. The median number of cycles of eribulin received was 3 (range 1-16 years). All patients, except one, discontinued treatment due to progressive disease and one patient due to adverse effects. Six patients had a dose reduction due to side effects. All patients had progressed at the time of the report with a median time to progression of 3 mo (range 1 to 14 mo). Fifteen patients had died with a median OS of 7 mo (range 1-18 mo). Six patients were alive with a median follow-up of 13.5 mo (range 7 to 19 mo).

**CONCLUSION:** This series of patients confirms the activity of eribulin in a heavily pre-treated metastatic breast cancer population consistent with phase Ⅱ and Ⅲ trials.

**Key words:** Breast cancer; Metastatic; Eribulin; Case series; Retrospective; Treatment



**Core tip:** This report discusses treatment with eribulin in clinical practice outside a clinical trial setting. It confirms the activity of eribulin in a heavily pre-treated metastatic breast cancer population.

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## INTRODUCTION

Breast cancer is the most common female carcinoma and among the most frequent causes of cancer mortality in women worldwide[1,2]. Metastatic breast cancer is considered incurable with an estimated median survival of 2 to 3 years and only 1 in 4 to 5 patients alive at 5 years after diagnosis. Treatment with endocrine, cytotoxic or targeted therapies has prolonged survival and may improve or maintain the quality of life of patients living with metastatic disease. Despite the availability of several active agents, there is a need to develop additional drugs in order to increase the treatment options in metastatic breast cancer patients.

Eribulin (previously known as E7389) is a new chemotherapy drug and has been approved by regulatory authorities for the treatment of metastatic breast cancer[3]. It is a microtubule inhibitor and a synthetic analog of the natural product halichondrin B isolated from the marine sponge *Halichondria okadai*. Its mechanism of action is unique among microtubule poisons and involves binding to polymerized tubulin without affecting de-polymerization and promoting tubulin sequestration in non-functional polymers[4]. It produces cell cycle arrest in the G2-M phase *in vitro* in several human cancer cell lines, including p-glycoprotein-expressing and paclitaxel-resistant lines, when incubated in the presence of nanomolar concentrations of the drug. Clinical development has led to the approval of eribulin for use as monotherapy in metastatic breast cancer patients who have received at least two prior chemotherapeutic regimens including an anthracycline and a taxane. This is consistent with the view that sequential monotherapies are preferable to combination therapies in most clinical scenarios in metastatic breast cancer as a means of preserving tolerability without compromising efficacy.

This report presents retrospective data on this drug in patients treated in two centers outside of a clinical trial.

## MATERIALS AND METHODS

Archives of all patients treated for metastatic breast cancer from 2011 to June 2014 were reviewed and 21 patients treated with eribulin were identified. Information on patients' characteristics and treatment outcomes was extracted. Data on response, progression-free survival (PFS) and overall survival (OS) were collected.

Treatment with eribulin mesylate was initiated at the recommended dose of 1.4 mg/m$^2$ on days 1 and 8 of a 21-day cycle in 17 patients and at a decreased dose of 1.1 mg/m$^2$ on days 1 and 8 of a 21-day cycle in 4 patients due to comorbidities and frailty. Tumor response was reported according to the revised Response Evaluation Criteria in Solid Tumors criteria[5]. Response rate was defined as the addition of complete response and partial response (PR). Disease control rate (DCR) was defined as the response rate plus the stable disease (SD) rate. PFS and OS are calculated by the Kaplan-Meier method starting from the date of eribulin therapy initiation to the date of disease progression documentation or death. Descriptive statistics parameters used in the study were performed with the aid of a non-commercial statistical calculation site (www.statpages.org).

Data collection and recording were conducted in compliance with the ethical requirements of our centers and patients' anonymity was guaranteed. Given that the study was retrospective in nature and the fact that treatments had been provided according to standards of care, no specific informed consents were obtained from individual patients.

## RESULTS

The median age of patients at the time of eribulin treatment was 53 years (range 34-75 years) (Table 1). The median age at the time of breast cancer diagnosis was 47 years (range 30-73 years). Nineteen patients were diagnosed initially with localized disease and had received adjuvant treatments and 2 patients had metastatic disease at diagnosis. Sixteen patients had Estrogen Receptor (ER) and/or Progesterone Receptor

**Table 1 Characteristics of patients and tumors in this series**

| | Median | Range |
|---|---|---|
| Age at diagnosis | 47 | 30-73 |
| Age at eribulin treatment | 53 | 34-75 |
| | Patients ($n$ = 21) | % |
| Hormonal status | | |
| ER/ PgR positive | 16 | 76.5 |
| ER/PgR / Her2 negative | 5 | 23.8 |
| Grade | | |
| Ⅲ | 12 | 57.2 |
| Ⅱ | 5 | 23.8 |
| Unknown | 4 | 19 |
| Performance status | | |
| 0-1 | 13 | 61.9 |
| 2-3 | 8 | 38.1 |
| Number of metastatic sites | | |
| 2 | 3 | 14.3 |
| More than 2 | 18 | 85.7 |

ER: Estrogen Receptor; PgR: Progesterone Receptor; Her2: Her2/neu oncogene of the EGFR (Epidermal Growth Factor Receptor) family.

**Table 2 Eribulin treatment and results**

| | Median | Range |
|---|---|---|
| Progression-free survival (mo) | 3 | 1-14 |
| Overall survival (patients who died) | 7 | 1-18 |
| Follow-up (mo, patients alive) | 13.5 | 7-19 |
| Number of eribulin cycles | 3 | 1-16 |
| | patients ($n$ = 21) | % |
| Previous lines of treatment | | |
| 3 or less | 8 | 38.1 |
| More than 3 | 13 | 61.9 |
| Reason for eribulin discontinuation | | |
| Progression | 20 | 95.2 |
| Adverse effects | 1 | 4.8 |

All patients in the series had progressed at the time of data collection. Fifteen patients had died at the time of data collection and 6 patients were still alive.

(PgR) positive disease and 5 had ER/PgR negative disease (all triple negative). Eight patients had received 2 or 3 previous lines of chemotherapy for metastatic disease and 13 patients had received 4 or more lines of treatment. The median number of previous lines of treatment was 4 (range 1-7). The median number of cycles of eribulin received was 3 (range 1-16). All patients had a heavy metastatic burden with two or more organs involved, including 5 patients with brain metastases and 10 patients with 4 or more metastatic organs.

All patients, except one, discontinued treatment due to progressive disease and one patient due to adverse effects (grade 3 asthenia) (Table 2). Six patients had a dose reduction due to adverse effects including febrile neutropenia, asthenia and peripheral neuropathy. Other grade 2 or 3 adverse effects observed included anemia in 2 patients and thrombocytopenia and ileus in one patient each.

Ten of 18 evaluable patients had a PR or SD for more than 3 mo as their best response resulting in a DCR of 55.6% (Table 3). Six of eight patients with 3 or less lines of previous therapies had a PR or SD resulting in a DCR of 75%, but some of the more heavily pre-treated patients (4 of 10, 40%) also benefited from eribulin treatment (Table 3). Three patients died before an evaluation of the effect of therapy could be performed. All patients had progressed at the time of the report with a median time to progression of 3 mo (range 1 to 14 mo) (Table 2). Fifteen patients had died with a median OS of 7 mo (range 1-18 mo). Six patients were alive with a median follow-up of 16 mo (range 7 to 19 mo). Kaplan-Meier PFS and OS curves are presented in Figures 1 and 2, respectively.

## DISCUSSION

The new chemotherapeutic agent eribulin has shown activity in metastatic breast cancer and represents a new therapeutic option in this disease after failure of other therapies such as anthracyclines and taxanes[4].

Phase Ⅰ studies have established the optimal three-weekly dose to be 1.4 mg/m$^2$ on days 1 and 8 which has been carried out for further development[6]. Phase Ⅱ and Ⅲ studies have established the efficacy of eribulin in pre-treated metastatic breast cancer patients. Two phase Ⅲ trials have compared eribulin to either physician's choice or a capecitabine arm[7,8]. The first, the Eisai Metastatic Breast Cancer Study Assessing Physician's Choice versus E7389 open label, randomized, multicenter trial, included 762 patients who had received at least 2 previous lines of chemotherapy and were randomized in a 2:1 fashion to receive either eribulin or a treatment of their physician's choice[7]. The control arm was designed to reflect various practices across continents. Almost all patients had received anthracyclines and taxanes and three fourths had also received capecitabine before study entry. The study showed a statistically significant benefit in OS for patients receiving eribulin treatment with an OS of 13.1 mo *vs* 10.6 mo [7]. No PFS benefit was demonstrated in the intention-to-treat analysis as per the independent review. Median PFS was 3.7 mo in the eribulin arm. Hematological toxicities, asthenia/fatigue and peripheral neuropathy were the most common toxicities in the eribulin arm. The second phase Ⅲ trial was recently published and 1102 patients, who had previously received anthracyclines and taxanes, were randomized in a 1:1 ratio to eribulin or capecitabine[8]. The trial showed eribulin and capecitabine to be equally effective for both PFS and OS[8]. Median PFS was 4.1 mo in the eribulin arm in this study. Pooled results of these two randomized phase Ⅲ studies have confirmed a statistically significant OS benefit in the eribulin arm with a median OS of 15.2 mo *vs* 12.8 mo in the control arm[9].

Interestingly, in the above pooled study, an a posteriori sub-group analysis showed that the eribulin OS benefit was consistent in all sub-types with the triple negative patients obtaining a slightly greater benefit from eribulin compared to control treatments[9]. In a

**Table 3 Clinical response rates of evaluable patients with metastatic breast cancer treated with eribulin *n* (%)**

| | All evaluable patients in the series (*n* = 18) | 95%CI | 3 or less previous lines of therapy (*n* = 8) | 95%CI | More than 3 previous lines of therapy (*n* = 10) | 95%CI |
|---|---|---|---|---|---|---|
| CR | 0 | 0 | 0 | 0 | 0 | 0 |
| PR | 7 (38.9) | 16.4-61.4 | 4 (50) | 18.7-81.8 | 3 (30) | 1.6-58.4 |
| SD | 3 (16.7) | 0-33.9 | 2 (25) | 0-55 | 1 (10) | 0-28.6 |
| PD | 8 (44.4) | 21.5-67.4 | 2 (25) | 0-55 | 6 (60) | 33.2-86.8 |

CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

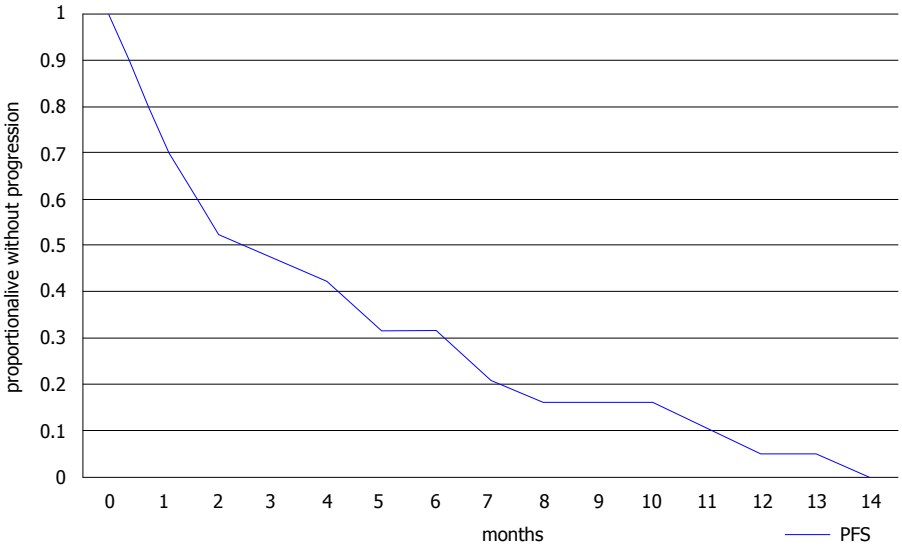


**Figure 1 Kaplan-Meier Progression-Free Survival curve of the patients in this series.** The Y axis depicts the percentage of patients alive without disease progression and the X axis represents months from the start of eribulin treatment. PFS: Progression-Free Survival.

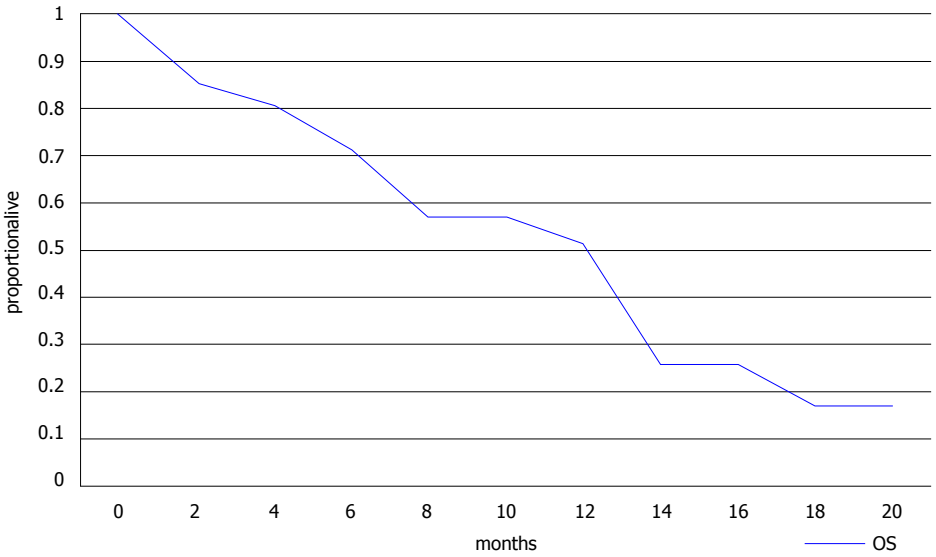


**Figure 2 Kaplan-Meier curve of Overall Survival of the patients in this series.** The Y axis depicts the percentage of patients alive and the X axis represents months from the start of eribulin treatment. OS: Overall survival.

retrospective study that included 133 patients, the clinical benefit of eribulin, especially in Her-2 negative patients, was confirmed[10]. In the first-line metastatic setting, a phase Ⅱ trial that included only Her-2 negative patients (both Hormone Receptor positive and triple negative) outlined the activity of eribulin in these patients which resulted in a median PFS of 6.8 mo[11].

Our small retrospective analysis confirmed that approximately 50% of patients were alive at 13 mo as shown by the Kaplan-Meier curve (Figure 2). Median

PFS was 3 mo and median OS was 7 mo in this heavily pre-treated population with a median of 4 previous lines of treatment (range 1 to 7). These results agree with those from the randomized trials and suggest that useful clinical activity can be expected in some patients who have received more than 3 previous lines of chemotherapy. The adverse effect profile of eribulin was also consistent with published data, with asthenia, neutropenia and peripheral neuropathy being the most common adverse effects. The patient population in our study was relatively young and only 3 patients were older than 65, however, studies have shown the feasibility of eribulin treatment in older patients[12].

Eribulin is added to the armamentarium of drugs with activity that may be used in metastatic breast cancer after anthracycline and taxane progression which may also include capecitabine[13], vinorelbine[14] and gemcitabine[15]. Nevertheless the short PFS highlights the need for more effective treatments or combinations.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Eribulin is a synthetic analog of a natural marine product and is a new chemotherapeutic approved for the treatment of metastatic breast cancer.

### Research frontiers

This report discusses treatment with eribulin in clinical practice outside a clinical trial. It confirms the efficacy of the drug in the day to day clinical setting.

### Innovations and breakthroughs

This is a confirmatory report of the usefulness of eribulin mesylate for advanced metastatic breast cancer.

### Applications

Eribulin is an additional option for the treatment of patients with metastatic breast cancer who have already received anthracyclines, taxanes and capecitabine.

### Terminology

Eribulin (previously known as E7389): A chemotherapy drug which is a microtubule inhibitor and a synthetic analog of the natural product halichondrin B isolated from the marine sponge *Halichondria okadai*.

### Peer-review

The paper described the observed outcomes of Eribulin treatment on a few breast cancer patients, it is a well written paper with a clear information on the outcomes of the treatment. It is a topic of interest to the journal's readers.

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