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Contents

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Contents

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REVIEW

Transition of Thalassaemia and Friedreich ataxia from fatal to chronic diseases

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Abstract

Thalassaemia major (TM) and Friedreich's ataxia (FA) are autosomal recessive inherited diseases related to the proteins haemoglobin and frataxin respectively. In both diseases abnormalities in iron metabolism is the main cause of iron toxicity leading to increased morbidity and mortality. Major efforts are directed towards the prevention of these diseases and also in their treatment using iron chelation therapy. Both TM and FA are endemic in Cyprus, where the frequency per total population of asymptomatic heterozygote carriers and patients is the highest worldwide. Cyprus has been a pioneering nation in preventing and nearly eliminating the birth of TM and FA patients by introducing an organized health structure, including prenatal and antenatal diagnosis. Effective iron chelation therapy, improved diagnostic methods and transfusion techniques as well as supportive therapy from other clinical specializations have improved the survival and quality of life of TM patients. Despite the tiresome clinical management regimes many TM patients are successful in their professional lives, have families with children and some are now living well into their fifties. The introduction of deferiprone led to the elimination of cardiac failure induced by iron overload toxicity, which was the major cause of mortality in TM. Effective combinations of deferiprone with deferoxamine in TM patients caused the fall of body iron to normal physiological ranges. In FA different mechanisms of iron metabolism and toxicity apply to that of TM, which can be targeted with specific iron chelation protocols. Preliminary findings from the introduction of deferiprone in FA patients have increased the hopes for improved and effective therapy in this untreatable condition. New and personalised treatments are proposed in TM and FA. Overall, advances in treatments and in particular of chelation therapy using deferiprone are transforming TM and FA from fatal to chronic conditions. The paradigm of Cyprus in the prevention and treatment of TM can be used for application worldwide.

Key words: Thalassaemia; Friedreich ataxia; Prenatal diagnosis; Survival; Chelation therapy; Deferiprone; Deferoxamine; Cyprus

Core tip: Thalassaemia major (TM) and Friedreich's ataxia (FA) are inherited diseases related to iron toxicity, with high morbidity and mortality rates. Cyprus has the highest frequency of TM and FA worldwide. Prenatal diagnosis and other health policies almost abolished the birth of TM and FA patients in Cyprus. Deferiprone has increased the survival and quality of life of TM patients, who are now reaching normal life expectancy and it is also promising for FA patients. Personalised treatments are proposed for TM and FA. The Cyprus experience can be used as a paradigm for the prevention and treatment of TM worldwide.

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INTRODUCTION

Thalassaemia major (TM) and Friedreich's ataxia (FA) are autosomal recessive inherited diseases with serious pathological complications, morbidity and mortality. Although the two diseases are genetically different they are both related to abnormalities in proteins of iron metabolism namely frataxin in FA and haemoglobin in TM[1-5].

Adult haemoglobin is composed of two alpha and two beta globin chains, each containing an iron molecule embedded in a protoporphyrin ring, which is responsible for the transport of oxygen to all cells of the body[1]. Frataxin is a mitochondrial matrix protein which functions in iron-sulfur cluster containing enzymes within the chain assembly responsible for respiration and energy transduction[3,4].

While frataxin is encoded by the gene of chromosome 9, the beta globin chains of haemoglobin are encoded by a single gene on chromosome 11 and the alpha globin chains of haemoglobin are encoded by two genes which are closely linked on chromosome 16[1,3,4].

In patients with TM, insufficient or no beta globin chains of haemoglobin are produced and the abnormal haemoglobin cannot deliver oxygen efficiently to the tissues. TM is a fatal disease if it is not treated with regular blood transfusions every 1-4 wk and chelation therapy[5].

FA is a progressive neurodegenerative disease with significant morbidity and has no effective treatment[6]. In FA patients the production of frataxin is severely reduced. Frataxin is a highly conserved mitochondrial matrix protein composed of 130 amino acids, has MWt 14.2 kDa and weakly binds iron[3,4]. In almost all FA patients there is an expansion of the guanine-adenine-adenine (GAA) trinucleotide in the first intron of both alleles of the frataxin gene. While in normal individuals the alleles of the frataxin gene have 36 or fewer GAA repeats, in FA disease the alleles have approximately 70 to more than 1200 to 1700 GAA repeats[3,4,6].

Major efforts have been taken worldwide for the control and reduction of births of TM and FA patients. Prenatal and antenatal diagnoses are increasingly being used in certain endemic areas and in ethnic groups for the prevention of these and other inherited diseases. Cyprus has the highest rate of asymptomatic heterozygote carriers and patients per population of both TM and FA by comparison to any other nation[7-11]. There is a 25% chance of TM or FA child being born from a pairing of asymptomatic heterozygote carrier parents. Within this context an effective prevention programme is needed for the containment of TM and FA in endemic areas like Cyprus.

Regarding patient treatment, recent developments involving primarily iron chelation therapy using the iron chelating drug deferiprone (L1) suggest that major advances have taken place in reducing morbidity and mortality in TM and also promising steps towards improvement in the treatment of FA[5,12]. The strategy responsible for these advances and the transition of these diseases from the fatal to the chronic stage can better be described using the paradigm of Cyprus[9]. Within this context the recent developments in the past few years in TM and FA in Cyprus have been reviewed with particular emphasis on the strategies regarding the health organisation structures, educational efforts, prevention measures and the overall clinical management. Related epidemiological and socioeconomic consequences are also described.

Special emphasis is also given to the mechanisms and pathogenesis of gross or focal iron overload toxicity in both TM and FA[5]. The treatment of gross or focal iron overload toxicity using chelation appears to be the major therapeutic option for decreasing the associated high mortality and morbidity observed in these two categories of iron metabolic diseases. The role of L1 is highlighted, since it is considered as one of the major factors in the transition of TM from a fatal disease to a chronic disease and also increases the prospects of effective treatment in FA[5].

EPIDEMIOLOGY, TREATMENT AND SURVIVAL OF THALASSAEMIA PATIENTS

TM is classified as a haemoglobinopathy, the most common group of inherited diseases in humans. More than 200 mutations of the haemoglobin genes have been reported, causing a range of pathological abnormalities from asymptomatic to fatal states[1,2]. Patients with TM can only survive if they are regularly transfused with normal red blood cells from appropriate blood donors. Iron accumulated from regular long term red blood cell transfusions is toxic to many organs and becomes fatal unless removed by chelating drugs. Iron overload toxicity in TM has one of the highest metal related morbidity and mortality rates globally[13].

The geographic distribution and prevalence of thalassaemia is mostly in developing countries found in the Mediterranean, Middle East and South East Asia, where over 90% of thalassaemia patients are born. More than 100000 thalassaemia babies is estimated to be born annually and there are 100 millions of thalassaemia heterozygote asymptomatic carriers worldwide[2]. In India alone the annual birth rate of TM is estimated at 9000[14].

Thalassaemia is considered as an orphan disease in the European Union (EU), the United States and other developed countries due to the low number of patients compared to the total population, which is mainly Caucasian[2,15-17]. The treatment of TM patients in EU countries (*e.g.*, Cyprus, United Kingdom, Greece and Italy) is supported by the state, while in most developing countries insufficient funds are available for their treatment.

Organisation structure and collaborating bodies involved with the coordination for the prevention diagnosis and treatment of thalassaemia in Cyprus

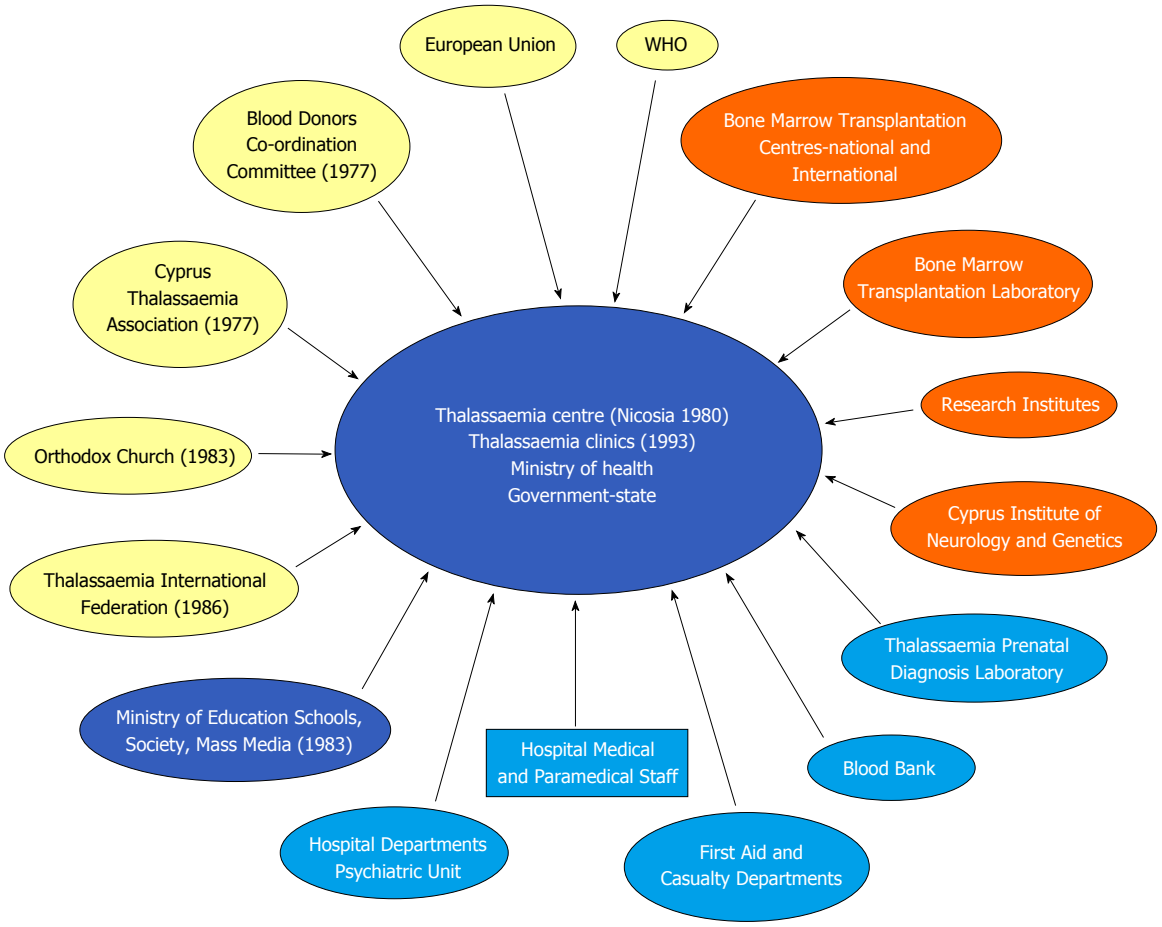

Figure 1 The organisational health structure and the chronological development of Thalassaemia in Cyprus. WHO: World Health Organization.

In the case of Cyprus, epidemiological data suggest that the indigenous population is numbered at around 800000 thousand and an extra 200000 thousand are mostly immigrants from developing countries working in Cyprus. The thalassaemia heterozygote frequency, which is associated mainly with the beta globin chain trait is 1 in 6 Cypriots. There are about 800 TM and thalassaemia intermedia patients ie (1 in 1000) who are regularly transfused and treated in specialist thalassaemia clinics in the main cities of Cyprus (Nicosia, Limassol, Larnaca, Paphos)[9].

Historically, the introduction of government policies to control thalassaemia came about after Cyprus gained independence in 1960. Before this period TM patients in Cyprus had a similar fate to TM patients in developing counties, where there is inadequate treatment including blood transfusions and chelation therapy[14,18,19].

Estimates from the health budgets for the treatment of TM patients were colossal for the size of Cyprus at the time of independence. Projections for the rate of birth of TM children and the associated expenditure on treatment predicted the collapse of the national health service or abandonment of the treatment due to unaffordable costs. The socioeconomic consequences for the families with TM children were devastating.

Based on these dilemmas the government of Cyprus introduced a new policy about 35 years ago for the set up of a thalassaemia centre, an educational campaign for population screening for thalassaemia trait, for the need of prenatal diagnosis of affected mothers, in addition to better clinical management for existing TM patients. An outline of the organization health structure, the collaborating bodies involved in the prevention, diagnosis and treatment of thalassaemia in Cyprus as well as a historic perspective is shown in Figure 1.

The plan for the prevention of births of TM babies includes the option of prenatal diagnosis, which provides an opportunity to the pregnant mother to decide about the course of the pregnancy, usually before the 3rd month of pregnancy. In the case of birth of a TM child, the survival prospects are directly related to the treatment options available at the country of residence. Non transfused TM patients die from ineffective erythropoiesis of normal red blood cells and related complications usually by the age of 2-7 years. If regular red blood cell transfusions are available from matched blood donors, survival is expected to increase to about 15-20 years. At this stage TM patients usually die from excess iron accumulation and iron overload toxicity induced organ damage. Cardiac failure due to excess iron deposition in the heart is the major cause of death in regularly transfused TM patients

who do not receive adequate iron chelation therapy[20-23].

Life expectancy in TM increases following the application of effective chelation therapy within a period of a few years having started transfusions. A few compliant TM patients who adhered to the recommended protocol of sc deferoxamine (DF) (40-60 mg/kg per day, at least 5 d per week) were treated successfully since birth and have now exceeded 50 years of age. Compliance with sc DF for the majority of TM patients is poor and the average life expectancy is much shorter. Within this context, before deferiprone (L1) was introduced the mean life span of TM patients in the United Kingdom, was estimated to be 30-35 years[24]. In contrast, following the introduction of L1 in 1999, an improvement in compliance and efficacy as well as a reduction of cardiac deaths was observed in Cyprus and many other countries[25-29]. The prospects of compliance in relation to chelation therapy have also increased following the introduction of deferasirox (DFRA) in 2007. Chelation therapy with DFRA may mostly benefit TM patients who are intolerant or have other complications with both DF and L1 chelation therapy[18]. Several aspects are influencing the morbidity and mortality of TM patients worldwide including the efficacy, toxicity, availability and cost of all chelating drugs. In particular, access to these drugs may especially influence the overall survival of TM patients most of who live in the developing countries[18].

EPIDEMIOLOGY, TREATMENT AND SURVIVAL OF FRIEDREICH ATAXIA PATIENTS

FA is the most common autosomal recessive ataxia condition. It was identified by the German physician Nikolaus Friedreich in 1863 and only recently in 1996 the genetic mutation as well as the affected protein namely frataxin was identified and characterized[30-32]. This neurodegenerative inherited disease related to low production of frataxin appears to cause abnormalities in iron metabolism including the accumulation of iron in the mitochondria and consequential pathological complications of different severity in various organs. In particular, it causes progressive damage to both the central and peripheral nervous system resulting in a variety of symptoms ranging from gait disturbance and speech problems to diabetes and heart disease[3,4,33,34].

The geographic distribution and prevalence of FA is mainly in countries with Caucasian populations. It accounts for almost 50% of all the cases of hereditary ataxia. The FA carriers in Europe are estimated to be about 1:90, where in the United States it affects 1 in 50000 people. Apart from Europe and the United States, cases of FA have been recorded in the indigenous population of Mexico, Iran and India[35-37]. However, FA appears not to exist among the indigenous populations of sub-Saharan Africans, American Indians, Chinese, Japanese and Southeast Asians[38].

The FA carrier rate in Cyprus is estimated to be about 1:10, where in a cluster in the villages Kathikas and Arodhes in the Paphos district of Cyprus it is 1:5 or 1:6[10,39]. A total of 26 patients with FA were identified in Cyprus in a study in 2009[11].

There are individual variations in the onset and the severity of FA, which is characterised by progressive tissue and organ damage and significant morbidity. The onset of FA is early and almost always present before the age of 20 years. Loss of ambulation occurs about 15 years after the onset of the disease. More than 95% of patients are wheelchair bound by the age of 45 years. Frequent clinical findings of FA include the incoordination of limb movements, impairment of position and vibration, dysarthria, nystagmus, diminished or absent tendon reflexes, scoliosis, *etc*. In general, FA has progressive multipathological effects with a life span of approximately 30-40 years and the most frequently reported causes of death are diabetes mellitus and cardiomyopathy[3,4,33,34].

The socioeconomic consequences for the FA patients and their families and the costs associated with the treatment and care of these patients requires state intervention including steps for its prevention, diagnosis and treatment at the local level and worldwide. Within this context, preliminary scientific and epidemiological evidence prompted the introduction of government measures for the prevention or decrease in the number of births of FA patients in Cyprus similar to the adopted model of TM. These prevention measures were concentrated in the Paphos district where most of the patients and carriers of FA were previously identified and also to descendants from the Paphos district living elsewhere in Cyprus[11].

THE ROLE OF DIAGNOSIS AND PREVENTION OF THALASSAEMIA AND FRIEDREICH ATAXIA IN CYPRUS

Prenatal and antenatal diagnoses are widely used for the prevention of many inherited diseases with serious or fatal clinical complications, which are endemic in certain areas or ethnic groups. Both TM and FA belong to this category of diseases and Cyprus has the highest rate of asymptomatic heterozygote carriers of these diseases in comparison to any other nation. Within this context the prevention programme for TM started about 35 years ago whereas for FA it was recently adopted by the government in 2011 using the same guidelines as for TM.

In Cyprus the fate of TM patients prior to independence in 1960 was similar to that of a large majority of TM patients in the developing countries today, namely early death at 2-7 years due to lack of blood transfusions and other treatments. Following the independence of Cyprus and the initiation of a transfusion programme the survival rate of the TM patients increased. However, blood supplies were insufficient due to the high demand and the high annual birth rate of TM children at that period which was about 70 new cases per year[7].

The alarming rate of TM births and the socioeconomic consequences for the small population of Cyprus at that period prompted the initiation of new measures by the government in conjunction with institutions and organisations that could facilitate the process of prevention and the improvement of the treatment of patients.

Several initiatives including educational and publicity measures were taken to inform the general public of the campaign for the prevention of births of TM children and its implications in the families affected and society in general. Within this context a programme for the prevention of TM has been operating in Cyprus since 1973[7]. However, the major and most effective strategy for prevention was founded on thalassaemia trait compulsory screening for premarital couples. The strategy of compulsory screening was introduced in 1983, after an agreement between the government of Cyprus and the church, which carried out almost all weddings at that time. Accordingly a medical certificate was required by the church from all the premarital couples for confirming their thalassaemia status before proceeding to marriage.

In the meantime, the doctor managing the thalassaemia clinic where the diagnosis was carried out had to screen and inform the premarital couples of the possibility of having children with TM if they were both thalassaemia carriers and also to inform them of the available options to them including the option to decide the course of the pregnancy. Following marriage the prevention programme included genetic counselling and screening tests which were offered only for couples with thalassaemia trait, including chorionic villus sampling at about 11 wk of gestation of the pregnant mother[40,41]. If the test indicated a TM foetus, then the various options available were discussed and the final choice made.

Recent TM preventive measures for couples with thalassaemia trait included other possibilities, for example the pre-implantation diagnosis procedure[40]. This procedure was brought in to effect in Cyprus in the last 17 years and involves *in vitro* fertilisation for the analysis of early embryos and also the selection of an egg free of haemoglobin disorders. Following selection, further procedures include fertilisation of the egg in the laboratory and implantation into the womb[40,42]. Another possible option for future application is *in utero* allogeneic bone marrow transplantation.

The screening for thalassaemia trait is in most cases relatively simple and inexpensive, involving the electrophoresis of haemoglobin from blood samples[43-46]. Similarly, a blood sample is used in the case of FA screening but a more expensive DNA analysis is required to identify an FA carrier[47-49]. The strategy adopted for screening for the FA trait status involves in the first instance only persons with origins from the areas in Paphos where high prevalence of FA was identified[11].

The screening for thalassaemia carriers and the introduction of prenatal and antenatal diagnosis resulted in a significant drop in the number of births of patients with TM. The birth rate of TM children in Cyprus between 1970 and 1980 was estimated to be about 30-50 per year and a total of about 400 TM births were recorded. Following the implementation of the government and church policy on thalassaemia in 1983, the birth rate of TM babies between 1984 and 1994 dropped to about 25, *i.e.*, a rate 2-3 per year[7-9].

However, new trends have arisen in the last 20 years in Cyprus including major demographic changes, which had a major influenced in the birth rate of TM patients. It is estimated that 20% of the people residing in Cyprus are foreign workers and the total population has increased to approximately 1 million. Cyprus became a member country of the EU in 2004 and as a result of implementation of EU laws a medical certificate for thalassaemia is not obligatory for civic weddings, unlike church weddings[9]. Similarly, many couples are deciding to have TM children instead of abortion, because of emotional, religious and ethical reasons and also as a result of the newly improved treatments, which lead to an increase in the life expectancy of TM patients. As a result of these trends and also the marriage of Cypriots with non-Cypriot carriers the number of births of TM children has increased slightly in the last few years[50]. It is estimated that there were about 25 births of TM children from 2005 to 2011, *i.e.*, a rate of 3-4 per year. The trend on the birth rate of TM patients is set to persist at present, unless the law on civic weddings is changed and one similar to that of church weddings is adopted[9]. In the meantime, further reduction in the birth rate of TM children in Cyprus is expected following the recent introduction of other techniques such as that of the pre-implantation diagnosis. This procedure is costly but allows the selection of a non TM foetus by parents with thalassaemia trait or by parents where one is a TM patient and the other has the thalassaemia trait[42].

The prevention programme for FA is similar to TM but with slight modifications. The major difference is that the screening programme for FA carriers is concentrated in premarital couples in the Paphos district and individuals living in other areas of Cyprus but originating from the Paphos district. In a previous study involving the Paphos district a total of 1050 persons aged 18 and over were screened and 98 (9.33%) FA carriers were identified[11]. More FA carriers have been identified since 2011, when the government prenatal screening programme for FA on premarital members of couples originating from Paphos was initiated. In this case the partners of those identified as FA carriers were also screened.

While major efforts have been taken in Cyprus for the diagnosis of carriers of TM and FA and the prevention of births of TM and FA patients, there are still a small number of births with these inherited diseases and especially of TM patients every year. The small number of births of TM patients is related to cases of non prenatal screening and the choice of parents to have a TM child. Within this context the treatment of the newly born and existing patients is another continuous challenge in the day to day running of the thalassaemia clinics and the

Table 1 Biochemical and clinical monitoring of Thalassaemia major patients

Determination of thalassaemia mutation and blood group (ABO, rhesus)
Vaccination: Flu, pneumonia, haemophilous influenza, meningitis a + c and hepatitis B
Cardiac: Physical examination, echocardiography, holter, stress test, muga scan, (once a year or earlier if required)
Liver and kidney function tests, urate, cholesterol, triglycerides, Ca, PO4, fasting glucose test and prothrombin time (every six months)
Blood glucose tolerance test and screening for hepatitis B antibodies, hepatitis C and HIV (every year)
Bone densitometry, chest X-ray, Endocrine (T3, T4, TSH, PTH), ophthalmology, ear, nose, throat and echo of the abdomen examination (every year)
Iron load estimation: Serum ferritin and Zn (every 3-4 mo), serum ferritin for patients with normal physiological iron range (every month), MRI T2 and T2* of the heart and liver (once a year)
Full blood count (every 1-1.5 wk for those treated with L1 and L1/DF combination)
Supporting specialist therapy team: Cardiologist, endocrinologist, haematologist, paediatrician, dietician, gynaecologist, psychologist. Other clinical faculties if required

The table was adapted from ref. [9]. HIV: Human immunodeficiency virus; MRI: Magnetic resonance imaging; DF: Deferoxamine; TSH: Thyroid-stimulating hormone; PTH: Parathyroid hormone.

government health structure adopted in general.

ORGANISED HEALTH STRUCTURES FOR THE TREATMENT OF THALASSAEMIA AND FRIEDREICH ATAXIA PATIENTS IN CYPRUS

The developmental model of an organised health structure for the prevention, diagnosis and treatment of TM in Cyprus was primarily based on an initiative of the parents of TM children and also the physicians in charge of the patients, other scientists, the church, the government and other non governmental organisations (Figure 1). The cornerstone of the health structure was based on the organisation of the Cypriot thalassaemia society and the blood donors association in 1977. Further milestones in the development of an organised health structure was the organisation in Nicosia (the capital of Cyprus) of the thalassaemia centre in 1980 and thereafter the gradual evolution of the thalassaemia clinics in each of the districts of Cyprus in 1993.

In the postnatal phase an organised health structure for the treatment of TM patients in Cyprus involving many health sectors and other organisations has been designed and developed over many years with the centre of activity based in the district thalassaemia clinics (Figure 1). The structure was based on the main form of treatment of TM patients, namely chronic red blood cell transfusions and iron chelation therapy. A continuous programme of biochemical and clinical monitoring involving many common laboratory tests and other clinical specialists was organised by the thalassaemia clinics (Table 1).

A major aspect of the treatment and the survival of TM patients is blood transfusion. Blood donation and collection in Cyprus is organized successfully by the volunteer blood donor co-ordination committee in conjunction with the blood bank of the ministry of health. The blood donors co-ordination committee in Cyprus was initiated in 1977 by the local thalassaemia society and a number of clinicians with the slogan "give blood and save lives" with a reference to the TM patients[7-9].

Blood donation and collection was coordinated and progressively covered all towns and villages in all districts and community sectors in Cyprus. It involved both state and private organisations such as the army, schools, universities, police, banks, businesses and many others. Blood donation is strictly on a volunteer basis and is organised and carried out in each district by the local blood donor coordination committee involving thousands of blood donors. It is a purely volunteer organization with no financial budget or treasurer. Blood supplies are generally sufficient but in the summer holidays for example problems may arise in blood donation and blood supply may be at a shortage. In the latter case the state media call for an emergency blood donation of the relevant blood groups in shortage to be donated at the nearest district hospital.

Continuous biochemical and clinical monitoring as well as supportive specialist care involving a multi-disciplinary team is available in all the thalassaemia clinics in Cyprus for the follow up treatment of TM patients (Table 1). The multi-disciplinary clinical team include almost all clinical sectors such as specialists in cardiology, endocrinology, haematology, paediatrics, nutrition, gynaecology, psychology, *etc*.[9].

Iron chelation therapy introduced within a few years of beginning the red blood cell transfusions is essential for the survival of TM patients. In the absence of iron chelation therapy the life expectancy of TM patients is less than 20 years. Under these circumstances TM patients usually die from excess iron accumulation and organ damage. In particular cardiac failure due to excess iron deposition in the heart is the major cause of death in regularly transfused TM patients who do not receive effective iron chelation therapy[20-24].

Iron chelation therapy is widely available for both the use of DF and L1 and also in many cases for DFRA in different countries including Cyprus[51]. The T2 and T2* relaxation time magnetic resonance imaging techniques have been used over the past decade for the routine diagnosis of excess iron load in the liver, the heart and also other organs[52-56]. Monitoring of serum ferritin levels is also routinely used for estimating body iron overload.

Organ function and other biochemical tests are also routinely performed at different intervals as shown in Table 1.

Bone marrow transplantation has also been considered as a therapeutic option especially for young patients (usually under 7 years) when a matched human leukocyte antigen sibling is identified by the bone marrow transplantation laboratory. It is estimated that a total of about 20 Cypriot TM patients received bone marrow transplantations and in most cases were successful except for one death and two graft rejections[9]. Similar results were reported in TM clinics in other countries[57-60].

Treatment problems and other complications associated with the life of TM patients are frequently assessed by arranging interviews between the clinician in charge of the thalassaemia clinic and each TM patient around every other week[9].

The small number of FA patients by comparison to the large number of TM patients in Cyprus required the adoption of a smaller different organisational health structure for treatment provision in FA. Despite the abscence of effective therapy in FA, major efforts are directed towards the therapy of the symptoms of FA patients. Clinical and biochemical monitoring, as well as supportive therapy for the FA patients is carried out in a specialised neurology centre in Nicosia the capital of Cyprus[11,39,61].

In relation to treatment it appears that gross body or focal iron overload toxicity is the main cause of death in both TM and FA. A better understanding of the normal iron metabolic pathways and the detection and characterisation of the iron abnormalities and iron overload toxicity in both TM and FA can lead to effective treatments and reduction of the morbidity and mortality in these diseases.

IRON METABOLISM IN NORMAL AND ABNORMAL CONDITIONS INCLUDING THALASSAEMIA AND FRIEDREICH ATAXIA

Iron is an essential metal required by all mammalian cells for many biological processes and normal physiological functions. The solubility of ferric (Fe^{3+}) iron in aqueous solution at pH 7.4 is negligible (10^{-18} mol/L) and iron precipitation rapidly occurs in biological media in the absence of chelating proteins or low molecular weight (LMwt) chelators. Ferric iron is deposited in cells in a polynuclear form as ferritin, which is water soluble and haemosiderin which is not water soluble. Ferrous (Fe^{2+}) iron is more soluble than ferric iron in physiological conditions. In biological systems ferrous iron is found in mononuclear form bound to proteins but not in polynuclear form as precipitates or deposits. Ferrous iron is a major catalyst of free radical reactions. In contrast, ferritin and other protein bound iron does not appear to facilitate the catalysis of free radical reactions. However, haemosiderin and haemosiderin-like ferric iron deposits (focal iron), as well as other forms of labile, LMwt ferric iron are considered to be toxic. The latter provide a continuous source of catalytic iron which under certain conditions can readily give rise to free radical reactions and cascades leading to oxidative stress damage.

There are many conditions associated with iron metabolic imbalance, which are caused by genetic, nutritional, iatrogenic, environmental, disease and other factors and their combinations[5,13]. These factors can cause deficiency or overload or abnormal distribution of iron and a corresponding pathological effect from the molecular to tissue levels[5,13,62]. Examples of such changes in iron metabolic disorders is nutritional iron deficiency anaemia, idiopathic haemochromatosis which is a genetic iron overloading condition, Hallervorden-Spatz syndrome where iron accumulation in the brain is observed, anaemia of chronic disease where iron is diverted and deposited in macrophages of the reticuloendothelial system, sideroblastic anaemia where iron is deposited in ring sideroblasts, FA where excess iron is deposited in mitochondria, atransferrinemia where the iron transport protein transferrin is not produced leading to abnormal iron distribution and many others[5,13]. Abnormalities in iron metabolism can also be observed in relation to changes in the many proteins involved in the regulation of iron metabolism and of changes involving iron containing proteins including the haemoglobinopathies[1,5,13].

Body iron levels and iron distribution in the organs are governed under normal conditions by homeostatic controls of iron uptake, distribution and storage. In general, these levels are regulated by dietary iron absorption in the gastrointestinal tract, the erythropoietic activity of the bone marrow and the rate of iron excretion[5,13,62]. The absorption of iron mostly takes place from the intestine and is regulated by several metabolic pathways involving regulatory proteins such as hepcidin and ferroportin[63]. Following the uptake of iron into the enterocytes from the gut lumen, iron is then taken by plasma transferrin and transported in blood for delivery to all the cells of all tissues[63,64].

The total body iron of normal adult humans is estimated at 3-5 g and is present in different forms mainly as haemoglobin (58%), which is found in red blood cells, myoglobin (9%) which is found in muscle tissue and also as intracellular ferritin/haemosiderin (30%) which is found mainly in the liver but also other organs (15-17). The uptake, transport and distribution of iron is tightly controlled. For example, the uptake of iron from transferrin by cells and its storage intracellularly is regulated by specific iron regulatory proteins based on the translational control of the transferrin receptor synthesis at the cell surface and also that of intracellular ferritin, as well as by hepcidin[5,13].

The amount of iron delivered to cells is mainly determined by the number of transferrin receptors and also the iron saturation of transferrin. Transferrin iron saturation under normal physiological conditions is 25%-35%. However, in transfusional iron overload or primary hae-

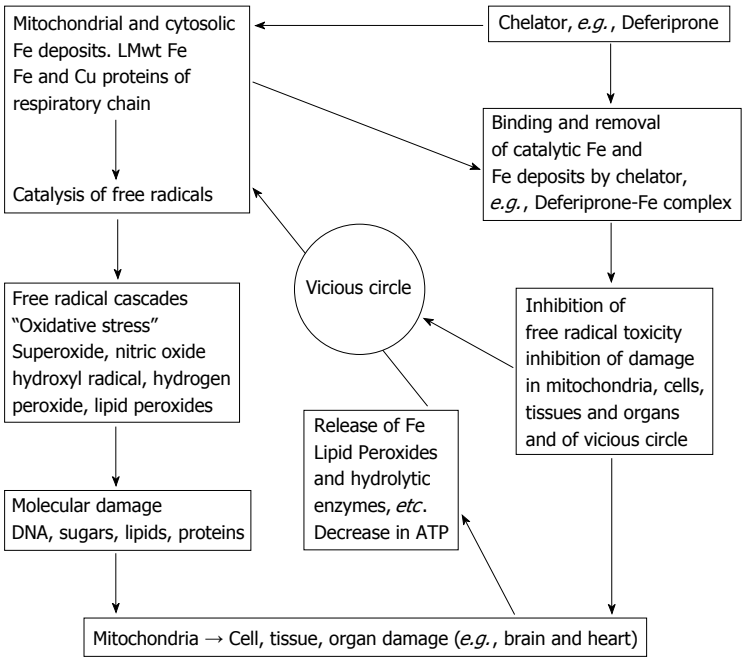

Figure 2 The mode of chelating and antioxidant activity of deferiprone in Friedreich Ataxia. Deferiprone can chelate intracellular and intramitochondrial iron deposits and labile low molecular weight (LMwt) iron, which are responsible for the catalytic formation of toxic free radicals and toxic byproducts. It can inhibit iron toxicity related damage to the heart and brain of Friedreich ataxia patients.

mochromatosis the saturation of transferrin with iron is much higher and usually exceeds 100%. At this stage where transferrin is saturated with iron, non-transferrin bound iron can be detected in the plasma[65-67].

The iron released from the continuous breakdown of effete red blood cells and the catabolism of haemoglobin, as well as of other cells of the body is transported in the blood by transferrin, which in conjunction with hepcidin and other regulatory proteins of iron metabolism maintains equilibrium between the sites of iron absorption from the gut, intracellular storage and also utilisation. Iron uptake by cells is accomplished by the binding of transferrin iron to a transferrin receptor on the cell surface. Usually two molecules of monoferric or diferric transferrin is bound to each transferrin receptor, which subsequently is incorporated into the cell within an endosome. The release of iron from transferrin is carried out through acidification of the endosome from pH 7.4 to 5.6. The iron molecules released are in a LMwt form and are incorporated in the "transit iron pool". Iron is then transported to all parts of the cell including the mitochondria, for storage in ferritin or haemosiderin and for incorporation into apoproteins for the formation of iron containing proteins.

The LMwt intracellular transit iron pool is thought to be composed mainly by LMwt chelator iron complexes[62,68]. Some LMwt naturally occurring chelators are found in cells, *e.g.*, citrate, ATP, ADP, glutathione, amino acids and some absorbed from food eg phytic acid, polyphenols and ascorbate. These and other similar molecules are thought to be involved in the transfer of iron in different intracellular compartments affecting intracellular iron metabolism. For example, they can form ternary metal complexes with apo-proteins or facilitate the incorporation of iron into the metal domain of proteins. The mechanisms and processes of iron uptake, release and exchange are governed by the same kinetic and thermodynamic parameters as for those applied to other chelators and their iron or other metal complexes[68]. Within this context, it is anticipated that chelators present at high concentrations eg citrate (10 mmol/L, in plasma) and glutathione in cells (5 mmol/L in liver cells) may play a significant role in these processes.

One of the most important functions of iron and some iron containing proteins is the catalysis of free radical reactions, and formation of related byproducts which are essential for physiological processes[69]. Free radicals and other nitrogen and oxygen activated products such as the nitrogen oxide, superoxide, hydroxyl radical, lipid peroxides and hydrogen peroxide are constantly and continuously generated in aerobic organisms during normal metabolism and physiological functions and also in response to both internal and external stimuli. However, if free radical reactions and related by-products are continuously overproduced and are not regulated or controlled by the antioxidant pathways and molecules, this can cause free radical toxicity and damage from the molecular level to the organ level as shown in many diseases including TM and FA[69-71].

The catalytic effects of iron can cause free radical toxicity cascades and biomolecular damage including damage to sugars, lipids, proteins, DNA and also widespread subcellular, cellular, tissue and organ damage (Figure 2). Such free radical cascades are not sufficiently neutralized by antioxidants but can usually be prevented by iron binding or chelation[69,70].

Iron absorption and transferrin iron delivery appears to be normal in FA patients, with the general iron metabolic parameters such as serum ferritin and transferrin iron saturation to be within the normal physiological ranges. The frataxin abnormality appears to affect selectively certain organs and tissues and the damage to be manifested slowly over many years, in a time scale similar to the case of iron overload in hereditary haemochromatosis patients[13,62,63]. In the affected cells the frataxin abnormality appears to cause the increased uptake and deposition of iron in mitochondria.

The transport of iron from the cytosol to the mitochondria and its incorporation into the apoproteins for the formation of mitochondrial iron containing proteins or incorporation into mitochondrial ferritin is not well characterised or understood in FA[72-74]. Similarly, there are controversies as to the presence of only mitochondrial iron deposits or both cytosolic and mitochondrial iron deposits in the affected cells. Accordingly, some investigators have reported the presence of excess iron in mitochondria but not the cytosol and others excess iron deposits in both sites[75,76].

A possible pathway can be suggested to explain these differences. Within this context and under normal conditions a pathway is operating involving the transport of LMwt iron complexes from the cytosol or protein bound iron, across the mitochondrial membrane and the formation of "intra-mitochondrial transit LMwt iron pool" before iron incorporation into the mitochondrial apoproteins or mitochondrial ferritin.

Incorporation of iron into ferritin including mitochondrial ferritin is usually in the reduced mononuclear ferrous form which is then oxidised and polymerised inside ferritin in an oxohydroxide iron phosphate polynuclear form[77,78]. Polynuclear iron cannot be incorporated inside ferritin but can be deposited as a haemosiderin-like polynuclear iron in the cytosol or mitochondria. In the latter case deposits of iron oxyhydroxy phosphate and sulphur polymers were identified in an animal model of FA, which were different from the forms of iron incorporated into mitochondrial ferritin[73]. Similarly, haemosiderin-like iron can also be formed from the breakdown of the ferritin protein shell and exposure of the intra-ferritin iron core[77,78]. Based on these observarions two different stages of iron deposition appear to be taking place in FA in a time dependent manner. In the initial stages there is an increased uptake of cytosolic iron into mitochondria, where cytosolic iron deficiency may also occur[75]. In the second stage, saturation of mitochondria with iron can cause progressive increase in cytosolic iron accumulation and the formation of iron deposits in the cytosol[76].

The iron deposition process in FA may be facilitated by several factors including the low production of ATP in mitochondria which decreases both the intra-mitochondrial LMwt transit iron pool and iron incorporation in mitochondrial ferritin, thus leading progressively to the increased polymerisation and deposition of haemosiderin-like iron in mitochondria[79]. Similarly, decreased production of ATP could cause a decrease in the cytosolic LMwt leading to progressive iron polymerisation and deposition in cytosol[78].

An additional factor that may facilitate excess iron deposition in mitochondria is the presence of oxidative environment which causes iron oxidation and precipitation. Ferrous iron is more water soluble than ferric iron and the former can be oxidised and precipitate forming a haemosiderin-like polynuclear iron inside the mitochondria. Other similar factors may possibly be involved in the intra-mitochondrial recycling of iron, where for example iron re-utilisation from the turnover of mitochondrial iron containing proteins is blocked and it undergoes polymerisation and accumulation as iron deposits in the mitochondria[80].

Another possibility for the excess iron deposition in mitochondria is the presence of a mechanism of iron loading of mitochondria similar to that observed in primary or hereditary haemochromatosis involving the malfunction of regulatory proteins like herceptin and ferroportin. In the case of chronic iron overload disorders, iron deposition progressively results in increased formation of haemosiderin polynuclear iron complexes which are difficult to mobilise. In such cases polynuclear iron removal from ferritin, haemosiderin and other forms of iron deposits follows the "last in-first out" general principle. Similarly, newly formed polynuclear iron formations and precipitates can be mobilised faster and more efficiently by chelation than older ones[81].

In addition to the toxic iron deposits and the increased oxidative stress observed in the mitochondria of FA patients, low frataxin levels can also cause other metabolic abnormalities such as the insufficient production of iron-sulfur clusters that are required for mitochondrial electron transport and energy transduction, as well as the functioning of aconitase and other iron containing enzymes or iron related metabolic pathways[79]. The distortion of these processes and in particular of mitochondrial electron transport is an additional powerful source of free radical production, which if not controlled can cause free radical cascades, toxicity and damage (Figure 2).

Organs such as the heart and the brain with high energy consumption and increased numbers of mitochondria appear to be particularly sensitive to these forms of abnormalities. Cardiac, brain and other organ damage, resulting in insufficient organ functioning levels is the overall cause of increased morbidity and mortality in FA.

PATHOGENIC EFFECTS OF THE IRON METABOLIC ABNORMALITIES IN THALASSAEMIA AND FRIEDREICH ATAXIA

There are many differences between TM and FA in relation to the iron metabolic pathways involved, such as the cellular and body distribution of iron, the origin and quantity of iron accumulated, the biochemical parameters

related to iron imbalance, the target organs of iron toxicity and many others. In assessing these differences it is important to specify some of the pathways, properties and characteristics of iron metabolism which are relevant to the diagnosis and treatment of each of these two conditions. Within this context the characterisation of the molecular pathways involved under normal physiological conditions and the pathological effects observed in both TM and FA, including the pathogenesis of gross or focal iron overload toxicity are considered essential in the understanding of the mechanisms involved in both diseases and also for the proposed treatment options.

Gross body iron overload is one of the most common metal overload toxicity condition which can be caused by increased iron absorption from the gut (primary haemochromatosis) or regular transfusions of red blood cells (secondary haemochromatosis) or a combination of both these two processes. Thalassaemia patients or patients with other refractory anaemias are regularly transfused every 1-4 wk, usually with 1-3 units (1 unit = 200 mg of iron) of red blood cells for maintaining haemoglobin levels above 9-10 mg/dL. Iron overload in refractory anaemias including TM has the highest morbidity and mortality rate worldwide in comparison to other iron or other metal overloading condition. One of the most seriously affected group of iron overloaded transfused patients are the TM patients.

The accumulated iron from transfusions cannot be excreted and is stored intracellularly as ferritin and especially as haemosiderin. The latter protein increases in concentration in some main organs, particularly in the liver as well as the heart and spleen of the transfused TM patients. Previous electron microscopy studies in cardiomyocytes and hepatocytes of TM patients have shown that in iron overloaded conditions iron loaded ferritin arrays are formed intracellularly mainly in primary lysosomes and haemosiderin iron aggregates in secondary lysosomes[21,82,83]. Further ultrastructural observations suggest that in heavy iron overload in TM there are some iron-laden lysosomes which are ruptured into the cell sap and may be a cause of toxicity. Other forms of subcellular damage which are identified for example in cardiomyocytes of TM who suffered congestive cardiac failure, include the presence of large cytoplasmic vacuoles, increase in the electron density of nuclei and increased amounts of heterochromatin, substantial loss of myofilaments, swollen mitochondria with loss of their cristae but with no iron deposits within them[84].

The damaging effects of iron overload and the rupture of iron-laden lysosomes into the cell sap can also cause the release of hydrolytic enzymes and potentially toxic forms of labile iron. The latter can catalyse the production of free radical cascades and cause further free radical damage which can progressively lead to a vicious cycle of further cellular, tissue and organ damage (Figure 2)[69,70].

Iron overload and toxicity causes organ damage, which is generally detectable when approximately 50-100 units of red blood cells have been transfused. This damage is reversible in the early stages but in many cases is so extensive that it can become irreversible[84]. Regularly transfused iron loaded TM patients usually die from iron overload related cardiomyopathy[20,23,26]. The possibility of congestive cardiac failure due to iron overload deposition in the heart is observed in TM patients with cardiac magnetic resonance imaging (MRI) T2* values of lower than 9 ms[53-55]. In addition to heart complications, other forms of iron overload toxicity include liver fibrosis and cirrhosis, splenomegaly, endocrine organ damage leading to diabetes, retarded growth and sexual immaturation. A major difference between TM and FA is that there is no iron accumulation in the brain or related toxic side effects involving the nervous system in TM patients.

Different molecular factors and pathological effects due to iron toxicity are observed in FA patients. Friedreich ataxia is an autosomal recessive disease like TM, which is caused by a mutation involving homozygous GAA trinucleotide intronic repeat expansions in the gene in chromosome 9, which encodes the mitochondrial matrix protein frataxin[3,4]. The repeat expansions interfere with transcription of the protein frataxin. About 97% of patients with FA have expansions of a GAA repeat in the first intron of both alleles of the frataxin gene. In normal individuals the alleles of the frataxin gene have 36 or fewer GAA repeats, while in the FA disease the alleles have from approximately 70 to about 1700 GAA repeats. It appears that regarding pathogenesis the age of onset correlates inversely to the size of the GAA repeat of the expansions, and directly with the rate of disease progression.

Frataxin appears to play an important role in mitochondrial iron homeostasis and is involved in the biosynthesis and structural assembly of iron-sulfur cluster proteins and also in haem synthesis. It is severely reduced to about 5%-20% of normal in FA patients but the level of its deficiency needed to cause disease is still unknown. The decreased production of frataxin in FA patients appears to be associated with many clinical abnormalities[3,4].

Variations in the levels of frataxin are observed between different tissues and the tissues mostly affected are expected to have severely reduced levels. A detailed description of the symptoms or signs and the anatomic localization involved is reviewed elsewhere[85]. The severity of the disease in FA and other inherited diseases seems to also depend on many other factors. Variations in compensatory mechanisms and alternative metabolic pathways which are in operation in affected cells and tissues may contribute to minimising the severity of FA[85]. Similar variations are observed in other inherited and metabolic diseases including TM and hereditary haemochromatosis[13,62,85-87].

Deficit of frataxin is mostly related to many abnormalities associated with mitochondrial structure and function. The main and characteristic abnormality in FA is an increased iron accumulation and deposition in the mitochondria matrix and a corresponding increase in

oxidative stress[3,4,88,89]. There is also deficit of mitochondrial respiratory chain complex activities and impairment of tissue energy metabolism, decrease in production of ATP, effects on the function of aconitase and other abnormalities which lead to mitochondrial misfunction, including also to a further increase in the production of free radicals and other reactive oxygen species. Increased cytosolic iron in cells of specific tissues is observed despite that the general body iron stores and metabolic parameters are within the normal physiological range in FA[90].

Histopathological and MRI studies have shown that iron primarily accumulates in heart muscle, dentate nuclei (spinocerebellar tracts) and spinal cord of patients with FA[76,91,92]. In these studies, focal localised iron deposits have been detected by MRI T2* in the brain and the heart of FA patients[76,91]. In histopathological studies stainable positive iron deposits have been identified in the cardiomyocytes in autopsy and biopsy specimens of FA patients[76]. It is important to note that patients with short GAA repeat expansions and long survival have neither heart disease nor focal localised iron deposits.

Many contributory factors appear to be involved in the observed variation in the onset and progression of disease in FA patients. In addition to the size of the GAA repeat expansions and the focal localised iron deposits in the heart and brain, other such factors include the possibility of frataxin isoforms, distribution of iron in other tissues or organs, dietary habits and many other[62,63,93]. The pathogenesis of the disease and the high level of toxicity observed mainly in the heart and the brain of FA patients may also be related to the high requirements and consumption but insufficient utilization of oxygen due to mitochondrial malfunctioning in these main target organs. Other organs appear not to be affected to the same extent because of reduced requirements, consumption and utilisation of oxygen.

There are many differences in the distribution and pathogenic effects of iron in TM and FA. In both conditions gross or focal iron overload deposits appear to be a major factor in the cause of the toxicity. Furthermore, in TM iron overload is in the form of ferritin and more so in the form of haemosiderin and situated in lysosomes in the cytosol and not the mitochondria[84]. In contrast, polynuclear iron deposits of mainly ferritin and haemosiderin have been identified in both the mitochondria and cytosol of cardiac and other biopsy samples of FA patients[76,88,89].

Despite that cardiomyopathy is the main cause of death in both diseases, iron accumulation in the hearts of patients with FA is overall much lower in comparison to TM patients. However, cardiomyocytes in FA appear to be more susceptible to toxicity and damage arising mainly from focal iron deposition, mitochondrial iron overload and respiratory chain protein malfunction in comparison to mainly cytosolic gross body iron overload deposition and toxicity in TM.

Iron deposition in the pancreas may also be partly the cause of the increased incidence of diabetes in both the TM and FA categories of affected patients[34,94,95]. Pancreatic damage due to iron overload is well documented in TM and in some cases it may be reversible with chelation therapy at the early stages, whereas in FA it is still under investigation[96].

Iron deposition in the cerebellum in the brain of FA patients, as well as in other parts of the central and peripheral nervous system may play a major part in the cause of the observed pathology of FA and especially the ataxia[85]. Neurons appear to be more susceptible to iron toxicity than other cells. In contrast iron accumulation in the hypophysis (pituitary gland) in the brain of TM patients affects body growth and delays puberty. No other parts of the brain or of the nervous system in general appear to accumulate and deposit iron in TM and no neurological effects have been reported in severely iron loaded TM patients.

In general, the clinical problems arising from the abnormalities of frataxin are not equally affecting the FA patients or the various organs with the exception of the heart, the brain and other parts of the nervous system[97]. For example the serum iron and haemoglobin levels of FA patients appear to be within the normal physiological range despite that haem synthesis is carried out in mitochondria mainly in the bone marrow and spleen[90]. This suggests that the haemopoietic tissues are not affected or targeted by the toxicity mechanisms related to low levels frataxin and also of other related abnormalities including iron deposition observed in the mitochondria and cytosol of FA patients[97].

Overall, the iron deposition rate in the mitochondria of the tissues affected in FA patients appears to be very slow. Similarly, the process of iron toxicity may take years to reach the stage for pathological symptoms to be expressed and identified. Similar time scales are observed in the deposition of excess gross body iron load, as well as the expression of iron toxicity and pathological symptoms in hereditary haemochromatosis patients[13].

Whatever the rate or mechanisms of iron accumulation and deposition in TM and FA, it appears that gross body or focal localised iron overload is the major source of pathogenesis and the major factor of the increased morbidity and mortality observed in both diseases. Within this context iron chelation is considered at present as the first line therapy for the treatment of TM and FA patients.

THE ROLE OF DEFERIPRONE IN CHANGING THALASSAEMIA FROM FATAL TO A CHRONIC DISEASE

Regularly transfused TM patients not receiving iron chelation therapy have a poor prognosis and usually die from congestive cardiac failure and other iron overload complications by the age of 20 years[20-24].

There are three main iron chelating drugs which are regularly used for the treatment of transfusional iron overload in TM and other conditions namely DF, L1

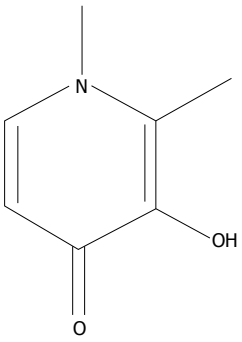

Figure 3 The chemical structure of the iron chelating drug deferiprone (L1). Deferiprone can bind iron through the two molecules of oxygen. It is a bidentate chelator and at physiological pH three molecules of L1 are used for binding one molecule of iron.

and DFRA. Both DF and L1 are generic drugs, whereas DFRA is a relatively new patented drug and all three are marketed in many countries worldwide[13].

Deferiprone was invented in 1981 in the United Kingdom and selected as a leading chelating compound out of about 150 related analogues and other chelating compounds (Figure 3)[13,98-101]. Many preclinical studies have shown promising results in the ability of L1 to mobilise iron from iron containing proteins, cells and animals and to prevent iron induced free radical toxicity[13,99-101].

The first clinical trials in iron loaded myelodysplasia and TM patients in 1987 in London, United Kingdom showed that oral L1 can cause equivalent iron excretion to subcutaneous DF[102,103]. The academic initiatives in the development of L1 continued and multicenter clinical trials were initiated by academic departments in many European countries, in Canada and in India, confirming the earlier results in London, United Kingdom[104-108]. The multicenter clinical trials involved mainly thalassaemia patients who were unable to receive DF due to toxicity, low compliance or both. Deferiprone was first registered in India in 1994 and then in the EU, Asian and other countries in 1999 and the United States in 2011[109].

The introduction of iron chelation therapy has increased the survival of TM patients. In a United Kingdom report prior to the introduction of L1 and DFRA the mean life expectancy of TM patients treated with DF was reported to be 35 years[24]. Deferoxamine was freely available to all TM patients in the United Kingdom and the main reasons of fatalities were iron overload cardiac failure due to non-compliance with the daily subcutaneous administration of DF and the inability of DF to remove effectively iron from the heart[24]. Similar outcomes with the DF treatment were observed in other countries such as Italy, Greece and Cyprus where DF was also freely available from the state to all TM patients[24-26].

Substantial reduction in morbidity and mortality has been observed since the introduction of L1 due to increased compliance and the effectiveness of L1 to remove excess cardiac iron. This improvement has been observed in many countries such as Cyprus, Greece, Italy, United Kingdom and Hong Kong where monotherapy of L1 or combinations with DF were used[25-29]. This was also encouraging evidence that iron overload toxicity is reversible and can be prevented in most cases especially when appropriate chelation therapy protocols using L1 and DF are introduced at an early age. Recent evidence suggests that the use of the appropriate chelation protocols and especially of specific combinations of L1 and DF could achieve the main aim of iron chelation therapy, namely the complete elimination of excess iron load and the associated iron overload toxicity complications[110].

However, the progress in the treatment of TM patients in developed countries including iron chelation therapy is not reflected in developing countries. It is estimated that more than 90% of TM patients worldwide, who mostly live in developing countries do not receive regular red blood transfusions and iron chelation therapy due to the high cost of treatment. Similar problems were encountered in Cyprus prior to 1977, when regular transfusions or chelation therapy were not accessible to the majority of patients due to the high cost, lack of facilities and lack of an organised health structure system.

Much progress has taken place in the treatment of TM and particularly regarding iron chelation therapy in the last 20 years in Cyprus that has changed the prognosis of TM from a fatal to a chronic disease. Since the introduction of L1 in Cyprus in 1999 many patients (70%) with poor compliance and adverse toxic effects to DF have either included L1 as a part of a combination protocol or switched over to L1 monotherapy[25]. The advances in chelation therapy with the introduction of L1 has led to the overall decrease in iron overload including serum ferritin levels, in improved compliance with chelation therapy, which was previously characterised as poor, as well as improved quality of life for patients[9,25].

The transition from a fatal to a chronic disease can be illustrated by the follow up of TM patients in the thalassaemia clinics of Cyprus. The TM patients undergo regular clinical and biochemical monitoring related to the complications of the underlying disease and the associated therapeutic applications including chelation therapy (Table 1)[9]. Similarly, the introduction of clinical studies and clinical trials involving TM patients in some of the clinics has played an important role in the development of general and personalised optimal therapies for the benefit of all patients[9,51].

Within this context clinical studies have shown that the most effective treatment of transfusional iron overload is the use of L1/DF combination protocols and particularly the International Committee On Chelation (ICOC) protocol (L1 at 75-100 mg/kg per day and DF at 40-60 mg/kg at least 3 d per week). This protocol appear to reduce serum ferritin and to increase MRI T2* of the heart and liver progressively to physiological normal levels[110-114]. Furthermore L1 monotherapy (85-100 mg/kg per day) appears to be sufficiently effective for maintaining the body iron stores at normal physiological levels in most TM patients[112,114]. This development is a major breakthrough in iron chelation therapy because it allevi-

ates TM patients from all the toxic side effects associated with iron overload toxicity and reduces substantially the overall morbidity and mortality to levels similar to those observed in the normal population[110,113].

An increase in compliance in TM patients was noted following the introduction of L1, in addition to the efficacy in iron removal. The majority of TM patients appear to receive satisfactory treatment with L1 in Cyprus either in combination with DF (> 50%) or as monotherapy (< 20%)[9,25]. Increase in compliance is also observed in TM patients receiving DFRA, despite that in some reports the efficacy in the overall iron removal and in particular iron removal from the heart is substantially reduced in comparison to the L1/DF combination[115,116]. Similarly, improved compliance and reduced episodes of local allergic reactions were also noted in many TM patients using an elastomeric pump as opposed to an electronic pump for the subcutaneous infusion of DF (< 20%)[9].

Important progress has also been observed following the introduction of the MRI T2* and T2 relaxation time techniques which have shown to be very valuable for monitoring excess iron load and for improving iron chelation therapy for the TM patients in Cyprus and other countries[9,52-56].

The most important finding in relation to chelation therapy in TM is that since the introduction of L1 the quality of life for most patients has greatly improved and most adult TM patients in Cyprus have developed professional careers, got married and had families. Characteristically about half of the 50 adult TM patients attending the thalassaemia clinic in Paphos have married, had in total 29 children and of note one female patient treated with L1 has successfully given birth to 4 children and also has two grandchildren[9].

The paradigm of Cyprus and the adopted organisational health structure for the prevention, diagnosis and treatment of TM including the protocols on iron chelation therapy with L1 in particular, could be used as an example to be followed by many countries where there is a high incidence of TM. Similarly, the high safety profile of L1 prompted its clinical application in many other non iron loaded conditions with focal localised iron deposits such as acute kidney disease, neurodegeneration with brain iron accumulation, Parkinson's disease and FA[117-120].

THE ROLE OF DEFERIPRONE IN THE TREATMENT OF FRIEDREICH ATAXIA

The general clinical care of FA patients is primarily concentrated on the treatment of the symptoms of the underlying disease. There is no established or effective treatment available for FA at present and no clear therapeutic targets or strategies developed to reverse the pathogenesis of the disease. Most efforts until recently were concentrated on the use of antioxidants for the treatment of the oxidative stress related pathology of FA. The most widely used antioxidants in FA are vitamin E, idebenone, coenzyme Q10, selenium and N-acetyl cysteine[121,122]. Despite that these antioxidants are used at random and in many cases for prophylaxis by FA patients the results are not encouraging and in most cases do not delay the onset or the progression of the disease[121,122].

The suggestion of the use of specific iron chelation therapy and especially L1 for the removal of excess iron from mitochondria for the prevention of pathogenesis and the treatment of FA in general was originally proposed in 2003[5]. The proposed mechanism for the mode of action of L1 was removal of localised excess deposited iron and inhibition of the iron induced catalytic activity and free radical cascades which caused increase in oxidative stress and cellular damage. This proposal was based on *in vitro* findings, animal studies and clinical results suggesting that L1 had access to all major organs and tissues including the heart and the brain[5,69,70]. The ability of L1 to remove excess deposited iron from the hearts of FA patients similar to TM patients, was also suggested since focal or gross iron overload appears to be the main cause of mortality in both diseases[5,69,70]. Similarly, it was also suggested that the application of L1 was a more appropriate therapy than the use of antioxidants for the toxicity arising from focal or localised excess deposited iron and also that the therapeutic options included drug combinations[5,69,70].

The suitability of L1 for clinical use in FA was until recently questioned, mainly because of lack of experience and knowledge on L1 chelation by those involved with the care of FA patients. Within this context a number of concerns were expressed regarding possible toxicities.

One of the major concerns in the use of iron chelating drugs in non iron loaded conditions including those with focal or localised excess deposited iron such as FA, Parkinson's disease, Alzheimer's disease and Hallevorden-Spatz syndrome is the induction of iron deficiency anaemia and other related toxicity. Despite that such hypotheses may be theoretically valid, each case should have been evaluated separately and background information on the clinical use of chelators thoroughly investigated. Within this context many wrong assumptions, expectations and conclusions can be made that may effect future investigations. In particular, the physicochemical, pharmacological and toxicological properties as well as the mode of action of chelators need to be taken into consideration for targeting each one of such conditions.

The specific properties of iron chelating drugs required for targeting iron toxicity and the risk/benefit assessment for use in each clinical condition have been previously reviewed[5,13]. Similarly, the suitability of L1 in FA and other non iron loaded conditions with focal or localised excess deposited iron as well as the concerns in relation to iron deficiency and related toxicity have also been previously addressed[5,69,70]. In such cases the interactions of chelators and especially L1 with tranferrin is essential in understanding the mechanisms and pathways involved[64,123].

In vitro and *in vivo* studies have previously shown that L1 can donate iron to unsaturated or partially saturated

tranferrin provided L1 is in the form of an iron complex and is present at a concentration of about the same level or lower than what is required to saturate tranferrin with iron[64,123]. Monitoring of plasma samples of normal individuals have shown that the oral administration of L1 causes a progressive increase of tranferrin iron saturation from the normal physiological range level (20%-35%) to a level of up to 80% over a period of 7 h[124]. This coincides with the results of pharmacokinetic studies where L1 has been shown to be cleared from plasma over a period of 6-7 h[67]. These findings suggest that L1 can mobilise iron from cytosolic intracellular storage sites or in the case of FA from focal iron deposits in mitochondria also and donate it to apotransferrin or monoferric transferrin in plasma, resulting in an increase of tranferrin iron saturation[64,124,125].

The above study suggests that the iron mobilised by L1 in individuals with normal body iron store levels including focal iron deposits in FA patients could be redistributed in the body *via* transferrin instead of being excreted. Measurement of urinary iron excretions have shown that usually only about 1-3 mg iron can be excreted by normal individuals of about 75 kg body weight, per a 3 g dose of L1[67,124-126]. In comparison, in iron loaded TM patients of approximately the same body weight, substantial increases in iron excretion of up to 71.5 mg can be observed by using the same dose of L1[67,123-126].

Despite that the net amount of iron excreted by L1 in normal individuals is small, continuous administration over prolonged periods of many months and years may cause iron deficiency, unless iron can be replaced from dietary sources or from iron supplements[64,123]. Monitoring of the biochemical parameters related to the iron status is particularly important for FA patients treated with L1 or other chelating drugs. The same monitoring applies for Parkinson's disease and other non iron loaded groups of patients, as well as for individuals using L1 as an antioxidant or anti-ageing drug, where administration of L1 may continue for many months and years[69,127].

Following the original proposal for the use of L1 in the treatment of FA several studies and clinical trials have been carried out confirming the selection and suitability of L1 as a promising therapeutic agent in this condition[5]. In cell studies using the HEK-293 FA model in which frataxin mRNA was knocked down, L1 (50 μmol/L) caused an increased in ATP production, mitochondrial membrane redox potential and resistance to staurosporine-induced apoptosis[128]. In the same model L1 outperformed DFRA, DF and salicylaldehyde isonicotinoyl hydrazone confirming its role as an effective iron chelator and antioxidant. The superiority of L1 in this model is based on its higher iron binding potential (log β3 = 36), the increased access to intracellular and intramitochondrial compartments, the iron mobilising and antioxidant properties and the efficient cellular exit of the L1 iron complex[13].

A number of clinical trials examining possible therapeutic effects of L1 in FA patients have been reported in the last few years. In one clinical trial nine Friedreich ataxia patients were treated with a dose of L1 of 20-30 mg/kg per day for 6 mo. In this study L1 has been shown to reduce excess toxic iron in the brain as determined by MRI T2*, and also to reduce ataxic gait and neuropathy in general[118]. Similar encouraging results were observed in patients with FA using a combination therapy of L1 with the antioxidant drug idebenone[129]. In another study 13 patients were treated with triple therapy using deferiprone at 5-25 mg/kg per day, idebenone at 10-20 mg/kg per day and riboflavin at 10-15 mg/kg per day for 15-45 mo[130]. Both neurological and heart function benefits were noted in some patients. Some adverse effects due to L1 were observed in four of the patients[130]. Furthermore, a double-blind, randomised, placebo-controlled phase 2 trial of L1 in a group of 80 individuals with FA was reported[6]. Full details of this study have not yet been published[6]. No significant overall changes in the ataxia scale scores in this study were observed, despite that improvement in posture, gait, and kinetic function were observed in some patients. Similarly, the L1 treatment was associated with cardiac improvement such as a decrease in the left ventricular mass. Decrease in serum ferritin levels was observed in a few patients. There was also one case of neutropenia which was resolved on L1 withdrawal[6].

Overall major therapeutic outcomes have been generally observed in FA patients treated with L1 during the clinical trials with both neurological and heart function improvements. It should be emphasised that complete clearance of excess cardiac iron in TM and brain iron in neurodegenerative disease patients has been previously achieved using higher doses of L1 over longer periods and the same result is expected in FA patients[112-114,119,131]. These optimistic results offer hope to FA patients who otherwise have no other effective treatment.

There are several concerns on the protocols used so far in FA patients including the low doses of L1 and the combinations with other antioxidant drugs. In particular, there is a scope of major improvements in the use of L1 in FA patients, including the prospects of personalised medicine. In the latter case the therapeutic targets have to be clearly defined and the appropriate L1 dose protocols selected based on the focal iron load in the brain, heart, pancreas and other organs, as well as the overall risk/benefit assessment in each patient. In general, the elimination of focal or localised iron deposits in the heart, the brain and other organs of FA patients are considered as a major therapeutic target in FA because it is a source of continuous toxicity and cause of pathological symptoms.

Previous clinical studies with L1 in non iron loaded patients or normal volunteers can serve as an example for designing appropriate treatment protocols of FA patients. For example, focal or labile iron deposits have also been targeted in kidney diseases patients. In clinical studies using doses of L1 of 50-75 mg/kg per day in about 50 non iron loaded patients with acute kidney disease for up to 9 mo, L1 improved kidney function, caused a de-

crease in proteinuria and no serious toxic side effects[117]. Overall L1 was well tolerated despite that the doses used were two or three times higher than those used in the FA studies. It is anticipated that similar higher doses of L1 at 50-75 mg/kg per day can also be used in most FA patients, especially those with increased focal iron deposits in the brain, heart and pancreas. The prospects of other improved therapies including chelator combination therapies, combinations with other drugs and the introduction of personalised medicine based on the focal iron deposits and the stage of the underlying disease should also be considered for future clinical investigations.

FUTURE PROSPECTS IN THE TREATMENT OF THALASSAEMIA AND FRIEDREICH ATAXIA

Major progress has been achieved in the diagnosis, prevention and treatment of TM and FA in the last two decades. The paradigm of Cyprus shows the way forward for the prevention and treatment of these pathological inherited diseases. However, these achievements have not yet been realised for most patients and in particular the vast majority of TM patients who live in developing countries. Similar developments have occurred in FA, but the lack of clear approaches and suitable strategies may undermine the efforts for prevention and effective therapeutic interventions, especially in relation to the removal of focal toxic iron deposits in the heart and the brain.

One of the major achievements of the last two decades is the substantial reduction in the births of TM children in Cyprus and many other countries worldwide using similar birth prevention measures. Public education policies and especially consultations with the heterozygote couples at risk of giving birth to TM children played a major role in this achievement. Similarly, the availability of prenatal and antenatal diagnoses substantially reduced the rate of births of TM children.

The quality of life and survival of TM patients have increased substantially in the last 20 years as a consequence of the introduction of effective therapeutic postnatal measures and especially diagnostic advances in the estimation of iron overload using the MRI T2* and T2 techniques and especially the effective treatment of iron overload with L1 and its combinations[86,132]. These changes and related improvements are more obvious in Cyprus and other countries where government health authority intervention is significant and appropriate by-laws as well as appropriate procedures are implemented using effective organisational health structures.

The organisational health structure adopted in Cyprus led to the decrease and almost the total elimination of births of TM children. The birth of a few TM children in Cyprus by those not adhering to the prenatal and antenatal screening programmes has highlighted the importance of the implementation of health policies related to the government's strategy for the prevention of thalassaemia. The introduction of other interventions such as preimplantation diagnosis has also shown in many cases an extra effective measure in reducing the birth of TM children[42]. However, it is a more difficult and risky procedure than prenatal diagnosis with low success rate and is also more expensive. The prospects of use of *in utero* allogeneic bone marrow transplantation in the future may decrease further the birth of TM children but this still requires further research[60,133].

The treatment of TM patients is also entering a new phase. Bone marrow transplantation offers a cure for TM patients but the risk of rejection of the transplant and of fatalities is still high[57-60]. The vast majority of TM patients follow the treatment of regular red blood cell transfusion and chelation therapy. Complete treatment of iron overload in TM patients is now envisaged from the use of the ICOC L1/DF combination protocol, which is leading progressively to the elimination of all excess toxic iron load and the attainment of normal iron levels, which are within the physiological range. This outcome increases the prospects of living free of iron related toxicity complications and with longer-term survival, similar to that observed in the general normal population[110,134].

In addition to the increased survival, the quality of life of TM patients has drastically changed in Cyprus and other countries adopting similar policies. The improvement in treatment, which mainly involved the introduction of L1 and other advances such as the introduction of elastomeric pumps for the delivery of subcutaneous DF and also the set up of a specialist multi-disciplinary team for the clinical follow up of TM patients has also contributed to this transition. For example, some of the TM patients have been attending tertiary education and many are employed in different professional sectors. In addition the marital and family status of the TM patients has also changed and most adults are married and have children. For example, in a fertility report of Cypriot TM women in 2004, improved attainment of reproductive capacity has been observed with a total of 358 successful pregnancies[134]. An increasing number of TM patients are now over 50 years of age and have become grandparents. This development followed the introduction of L1 and the L1/DF combination, where there was a significant drop in the mortality rate of TM patients from cardiac complications and also the reversal of iron overload toxicity in other organs[9,25-29,113,134].

The apparent increase in survival of TM patients is observed in Cyprus, and also many other countries using related chelation therapy and similar supportive therapy protocols[25-29]. The transition leading to the longer term survival outcome appears to be related to the improved compliance in the oral chelation therapy and more importantly to the ability of L1 to mobilise rapidly excess iron deposits from the heart, which has also been repeatedly confirmed in several thalassaemia centers using the T2* and T2 relaxation time MRI techniques[54,55,111,116].

The ability of L1 to reverse cardiac, liver, kidney and possibly other organ or tissue iron related damage in dif-

ferent categories of patients and its antioxidant properties provide further evidence of its potent therapeutic effects[69,70]. This therapeutic option may also apply to FA patients at present where so far no effective treatment has become available and L1 appears to offer the most promising therapeutic option, especially if used at the appropriate doses and protocols.

The exceptional therapeutic properties of L1 by comparison to other therapeutics in FA include several advantages such as its ability to enter most tissues and organs including the heart and the brain and also subcellular organelles such as mitochondria and cytosolic compartments at high therapeutic concentrations and to remove progressively and effectively toxic iron deposits. The efficacy of L1 in targeting iron toxicity arising from labile toxic iron forms and also from gross body or focal localised iron deposits, as well as its ability to redistribute iron *via* transferrin and reach iron balance in tissues, can make L1 a universal iron detoxifier and iron metabolic regulatory drug in many diseases including FA[64,69,117,135,136]. In addition, L1 can also act as an antioxidant by inhibiting iron catalysed free radical reactions and cascades arising from mitochondrial malfunction, which is considered a major cause of toxicity in the pathology of FA and also of other diseases (Figure 2)[5,69,70].

There are many other therapeutic advantages in the clinical use of L1 in FA and other conditions[64,69,70,137]. For example, the role of other metals in addition to iron in the cause and acceleration of neurodegeneration in FA and also other diseases is an expanding area under investigation[138-141]. Within this context, L1 can chelate and mobilise aluminium and copper and eliminate their associated toxicity as shown in cell and animal studies and also in clinical conditions[64,142-144].

Future prospects regarding the treatment of FA include the identification of all the molecular targets associated with the pathological mechanisms, the design of new therapeutics and protocols, as well as the optimisation of the existing experimental treatments. Within this context the importance of mitochondrial and cytosolic iron deposition and its significance in the neuropathy, cardiomyopahy as well as other organ damage needs to be further investigated, qualified, quantified and the associated toxicity defined similar to the iron overload toxicity in TM[54-56].

The optimisation of chelation therapy in FA needs to be further investigated. More effective removal of iron in FA can be achieved using higher doses of L1 than those used in the reported clinical trials. In this case two or three divided doses to a total of 50-75 mg/kg per day of L1 can be used depending on the level of focal iron deposition, which can be determined by the MRI T2 and T2* techniques[54-56]. Similarly, prophylactic use of lower doses of L1 (*e.g.*, 10 mg/kg per day) can also be used at the early stages in young diagnosed patients to delay or prevent focal iron load deposition and the onset of pathological symptoms.

Alternative and complimentary experimental treatments to that of iron chelation therapy could also be considered. For example, regular venesection treatment at an early age can be investigated in FA patients not only for prophylaxis but also as a form of main treatment at later stages. This treatment may be more appropriate for FA patients with excess mitochondrial and cytosolic iron deposition. Chronic venesection treatment is simple and inexpensive and can be well tolerated and used before and after the onset of the symptoms in FA patients. The rate of venesection can vary from that of regular blood donors to a more intensive one similar to that of hereditary haemochromatosis patients[13]. Reduction in the rate of organ and mitochondrial focal iron load deposition and associated toxicity as well as delay in the onset of pathological symptoms is expected using this form of treatment.

The therapeutic use of hyperbaric oxygen as a prophylactic measure in asymptomatic FA patients before the onset of mitochondrial focal iron load could also be considered for improvement of neuronal function[145]. Relative improvements were previously observed following the therapeutic use of hyperbaric oxygen in some stroke patients who had focal neuronal damage and substantial reduction of ATP production[145]. This method can also be applied to FA patients with focal iron deposits and pathological symptoms but special safety procedures may be required because of possible exacerbation of the mitochondrial induced oxidative stress. Combination therapies of chelating drugs, antioxidants, hyperbaric oxygen and venesection could provide an alternative investigational approach to the treatment of FA patients.

Gene therapy is the ultimate target for the complete treatment of both TM and FA[146,147]. However, such experimental therapies have been tried in many diseases with no optimistic outcome so far[146,147]. In all cases of the proposed introduction of new protocols or new treatments for FA or TM patients, a risk/benefit assessment is necessary for comparison of the existing and the new treatments. This assessment is particularly important for FA patients where no effective treatments are available at present.

The recent progress in the prevention, diagnosis and treatment of TM and FA patients in Cyprus can serve as a model by other countries and worldwide organisations such as the World Health Organization for minimising the births of TM and FA children and for improving the treatment of these and other inherited diseases worldwide. In particular, the organisational health structures adopted and the new treatments provided are shifting the disease profile of TM from a fatal disease to chronic one, in Cyprus and other countries using similar models. It is also hoped that the same model could be adopted for FA, including the introduction of effective new treatments.

Further efforts are needed for the development and improvement of organisational health structures for the prevention, diagnosis and treatment of TM and FA patients in developing countries, where health resources are extremely limited. Similarly, further advances in the de-

sign of personalised medicine which can provide optimal therapies for individual patients are also in progress and are the subject of in depth investigation.

CONCLUSION

The experience of the Cypriot model of organisational health structure implies that prenatal and antenatal diagnoses can be successfully used for reducing substantially the birth rate of TM and FA patients and the overall prevention of the incidence of these inherited pathological diseases worldwide. The introduction of new treatment methods, particularly the use of effective therapeutic protocols of L1, resulted in many improvements including better compliance with chelation therapy and improved the quality of life, as well as reduction or reversal of iron overload related organ damage and an overall increase in the survival of the TM patients in Cyprus and other countries using similar iron chelation protocols.

Similarly, the introduction of L1 in the treatment of FA patients increases the prospects of reducing the morbidity and mortality rates observed in this disease. New possible treatments for FA patients such as improved chelation dose protocols of L1, venesection and hyperbaric oxygen could be investigated and selected on individual patient cases based on a risk/benefit assessment. Improved diagnostic techniques for the detection of stored iron such as MRI and of iron toxicity could improve the therapeutic targeting methods for both TM and FA.

It appears that recent advances in the diagnosis of the pathogenesis and the treatment are changing TM and FA from fatal to chronic diseases.

REFERENCES

1 **Weatherall DJ**, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. *Bull World Health Organ* 2001; **79**: 704-712 [PMID: 11545326]

2 Community control of hereditary anaemias: memorandum from a WHO meeting. *Bull World Health Organ* 1983; **61**: 63-80 [PMID: 6601544]

3 **Koeppen AH**, Mazurkiewicz JE. Friedreich ataxia: neuropathology revised. *J Neuropathol Exp Neurol* 2013; **72**: 78-90 [PMID: 23334592 DOI: 10.1097/NEN.0b013e31827e5762]

4 **Payne RM**, Wagner GR. Cardiomyopathy in Friedreich ataxia: clinical findings and research. *J Child Neurol* 2012; **27**: 1179-1186 [PMID: 22764179 DOI: 10.1177/0883073812448535]

5 **Kontoghiorghes GJ**, Neocleous K, Kolnagou A. Benefits and risks of deferiprone in iron overload in Thalassaemia and other conditions: comparison of epidemiological and therapeutic aspects with deferoxamine. *Drug Saf* 2003; **26**: 553-584 [PMID: 12825969]

6 **Wilson RB**. Therapeutic developments in Friedreich ataxia. *J Child Neurol* 2012; **27**: 1212-1216 [PMID: 22791549 DOI: 10.1177/0883073812449691]

7 **Angastiniotis MA**, Hadjiminas MG. Prevention of thalassaemia in Cyprus. *Lancet* 1981; **1**: 369-371 [PMID: 6109998]

8 **Angastiniotis M**, Kyriakidou S, Hadjiminas M. The Cyprus Thalassemia Control Program. *Birth Defects Orig Artic Ser* 1988; **23**: 417-432 [PMID: 3390571]

9 **Kolnagou A**, Kontoghiorghes GJ. Advances in the prevention and treatment are changing thalassemia from a fatal to a chronic disease. experience from a Cyprus model and its use as a paradigm for future applications. *Hemoglobin* 2009; **33**: 287-295 [PMID: 19814674 DOI: 10.3109/03630260903212043]

10 **Dean G**, Chamberlain S, Middleton L. Friedreich's ataxia in Kathikas-Arodhes, Cyprus. *Lancet* 1988; **1**: 587 [PMID: 2894517]

11 **Zamba-Papanicolaou E**, Koutsou P, Daiou C, Gaglia E, Georghiou A, Christodoulou K. High frequency of Friedreich's ataxia carriers in the Paphos district of Cyprus. *Acta Myol* 2009; **28**: 24-26 [PMID: 19772192]

12 **Kontoghiorghes GJ**. Oral iron chelation is here. *BMJ* 1991; **303**: 1279-1280 [PMID: 1747667]

13 **Kontoghiorghes GJ**, Eracleous E, Economides C, Kolnagou A. Advances in iron overload therapies. prospects for effective use of deferiprone (L1), deferoxamine, the new experimental chelators ICL670, GT56-252, L1NA11 and their combinations. *Curr Med Chem* 2005; **12**: 2663-2681 [PMID: 16305464]

14 **Verma IC**. Burden of genetic disorders in India. *Indian J Pediatr* 2000; **67**: 893-898 [PMID: 11262988]

15 **US Department of Health and Human Services**. A Valuable Resource for Drug Developers: The Rare Disease Repurposing Database (RDRD). 2013. Available from: URL: http: //www.fda.gov/ForIndustry/DevelopingProductsforRareDiseasesConditions/Howtoapplyfor OrphanProductDesignation/ucm216147.htm

16 **Orphanet**. List of orphan drugs in Europe [Online]. Available from: URL: http: //www.orpha.net/orphacom/cahiers/docs/GB/list of orphan drugs in europe.pdf

17 **US Department of Health and Human Services**. Genetic and Rare Diseases Information Center. Available from: URL: http: //www.rarediseases.info.nih.gov/RareDiseaseList.aspx?StartsWith=A

18 **Kontoghiorghes GJ**. Ethical issues and risk/benefit assessment of iron chelation therapy: advances with deferiprone/deferoxamine combinations and concerns about the safety, efficacy and costs of deferasirox. *Hemoglobin* 2008; **32**: 1-15 [PMID: 18274978 DOI: 10.1080/03630260701726533]

19 **Teawtrakul N**, Chansung K, Sirijerachai C, Wanitpongpun C, Thepsuthammarat K. The impact and disease burden of thalassemia in Thailand: a population-based study in 2010. *J Med Assoc Thai* 2012; **95** Suppl 7: S211-S216 [PMID: 23130457]

20 **Zurlo MG**, De Stefano P, Borgna-Pignatti C, Di Palma A, Piga A, Melevendi C, Di Gregorio F, Burattini MG, Terzoli S. Survival and causes of death in thalassaemia major. *Lancet* 1989; **2**: 27-30 [PMID: 2567801]

21 **Kyriacou K**, Michaelides Y, Senkus R, Simamonian K, Pavlides N, Antoniades L, Zambartas C. Ultrastructural pathology of the heart in patients with beta-thalassaemia major. *Ultrastruct Pathol* 2000; **24**: 75-81 [PMID: 10808552]

22 **Aessopos A**, Farmakis D, Hatziliami A, Fragodimitri C, Karabatsos F, Joussef J, Mitilineou E, Diamanti-Kandaraki E, Meletis J, Karagiorga M. Cardiac status in well-treated patients with thalassemia major. *Eur J Haematol* 2004; **73**: 359-366 [PMID: 15458515]

23 **Kremastinos DT**, Farmakis D, Aessopos A, Hahalis G, Hamodraka E, Tsiapras D, Keren A. Beta-thalassemia cardiomyopathy: history, present considerations, and future perspectives. *Circ Heart Fail* 2010; **3**: 451-458 [PMID: 20484195 DOI: 10.1161/CIRCHEARTFAILURE.109.913863]

24 **Modell B**, Khan M, Darlison M. Survival in beta-thalassaemia major in the UK: data from the UK Thalassaemia Register. *Lancet* 2000; **355**: 2051-2052 [PMID: 10885361]

25 **Telfer PT**, Warburton F, Christou S, Hadjigavriel M, Sitarou M, Kolnagou A, Angastiniotis M. Improved survival in thalassemia major patients on switching from desferrioxamine to combined chelation therapy with desferrioxamine and deferiprone. *Haematologica* 2009; **94**: 1777-1778 [PMID: 19815834 DOI: 10.3324/haematol.2009.009118]

26 **Borgna-Pignatti C**, Cappellini MD, De Stefano P, Del Vecchio GC, Forni GL, Gamberini MR, Ghilardi R, Piga A,

Romeo MA, Zhao H, Cnaan A. Cardiac morbidity and mortality in deferoxamine- or deferiprone-treated patients with thalassemia major. *Blood* 2006; **107**: 3733-3737 [PMID: 16373663]

27 **Au WY**, Lee V, Lau CW, Yau J, Chan D, Chan EY, Cheung WW, Ha SY, Kho B, Lee CY, Li RC, Li CK, Lin SY, Ling AS, Mak V, Sun L, Wong KH, Wong R, Yuen HL. A synopsis of current care of thalassaemia major patients in Hong Kong. *Hong Kong Med J* 2011; **17**: 261-266 [PMID: 21813892]

28 **Voskaridou E**, Ladis V, Kattamis A, Hassapopoulou E, Economou M, Kourakli A, Maragkos K, Kontogianni K, Lafioniatis S, Vrettou E, Koutsouka F, Papadakis A, Mihos A, Eftihiadis E, Farmaki K, Papageorgiou O, Tapaki G, Maili P, Theohari M, Drosou M, Kartasis Z, Aggelaki M, Basileiadi A, Adamopoulos I, Lafiatis I, Galanopoulos A, Xanthopoulidis G, Dimitriadou E, Mprimi A, Stamatopoulou M, Haile ED, Tsironi M, Anastasiadis A, Kalmanti M, Papadopoulou M, Panori E, Dimoxenou P, Tsirka A, Georgakopoulos D, Drandrakis P, Dionisopoulou D, Ntalamaga A, Davros I, Karagiorga M. A national registry of haemoglobinopathies in Greece: deducted demographics, trends in mortality and affected births. *Ann Hematol* 2012; **91**: 1451-1458 [PMID: 22526366]

29 **Modell B**, Khan M, Darlison M, Westwood MA, Ingram D, Pennell DJ. Improved survival of thalassaemia major in the UK and relation to T2* cardiovascular magnetic resonance. *J Cardiovasc Magn Reson* 2008; **10**: 42 [PMID: 18817553 DOI: 10.1186/1532-429X-10-42]

30 **Campuzano V**, Montermini L, Moltò MD, Pianese L, Cossée M, Cavalcanti F, Monros E, Rodius F, Duclos F, Monticelli A, Zara F, Cañizares J, Koutnikova H, Bidichandani SI, Gellera C, Brice A, Trouillas P, De Michele G, Filla A, De Frutos R, Palau F, Patel PI, Di Donato S, Mandel JL, Cocozza S, Koenig M, Pandolfo M. Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science* 1996; **271**: 1423-1427 [PMID: 8596916]

31 **Dürr A**, Cossee M, Agid Y, Campuzano V, Mignard C, Penet C, Mandel JL, Brice A, Koenig M. Clinical and genetic abnormalities in patients with Friedreich's ataxia. *N Engl J Med* 1996; **335**: 1169-1175 [PMID: 8815938]

32 **Campuzano V**, Montermini L, Lutz Y, Cova L, Hindelang C, Jiralerspong S, Trottier Y, Kish SJ, Faucheux B, Trouillas P, Authier FJ, Dürr A, Mandel JL, Vescovi A, Pandolfo M, Koenig M. Frataxin is reduced in Friedreich ataxia patients and is associated with mitochondrial membranes. *Hum Mol Genet* 1997; **6**: 1771-1780 [PMID: 9302253]

33 **Weidemann F**, Störk S, Liu D, Hu K, Herrmann S, Ertl G, Niemann M. Cardiomyopathy of Friedreich ataxia. *J Neurochem* 2013; **126** Suppl 1: 88-93 [PMID: 23859344 DOI: 10.1111/jnc.12217]

34 **Cnop M**, Mulder H, Igoillo-Esteve M. Diabetes in Friedreich ataxia. *J Neurochem* 2013; **126** Suppl 1: 94-102 [PMID: 23859345 DOI: 10.1111/jnc.12216]

35 **Rasmussen A**, Gómez M, Alonso E, Bidichandani SI. Clinical heterogeneity of recessive ataxia in the Mexican population. *J Neurol Neurosurg Psychiatry* 2006; **77**: 1370-1372 [PMID: 17110750]

36 **Mukerji M**, Choudhry S, Saleem Q, Padma MV, Maheshwari MC, Jain S. Molecular analysis of Friedreich's ataxia locus in the Indian population. *Acta Neurol Scand* 2000; **102**: 227-229 [PMID: 11071107]

37 **Salehi MH**, Houshmand M, Aryani O, Kamalidehghan B, Khalili E. Molecular and clinical investigation of Iranian patients with Friedreich ataxia. *Iran Biomed J* 2014; **18**: 28-33 [PMID: 24375160]

38 **Labuda M**, Labuda D, Miranda C, Poirier J, Soong BW, Barucha NE, Pandolfo M. Unique origin and specific ethnic distribution of the Friedreich ataxia GAA expansion. *Neurology* 2000; **54**: 2322-2324 [PMID: 10881262]

39 **Christodoulou K**, Deymeer F, Serdaroğlu P, Ozdemir C, Poda M, Georgiou DM, Ioannou P, Tsingis M, Zamba E, Middleton LT. Mapping of the second Friedreich's ataxia (FRDA2) locus to chromosome 9p23-p11: evidence for further locus heterogeneity. *Neurogenetics* 2001; **3**: 127-132 [PMID: 11523563]

40 **Petrou M**, Ward RH, Modell B, Karagozlu F, Ozunlü VA, Hunt K, Old J, Weatherall DJ. Obstetric outcome in first trimester fetal diagnosis for the haemoglobinopathies. *Lancet* 1983; **2**: 1251 [PMID: 6139594]

41 **Petrou M**. Preimplantation genetic diagnosis. *Hemoglobin* 2009; **33** Suppl 1: S7-S13 [PMID: 20001634 DOI: 10.3109/03630260903344838]

42 **Kuliev A**, Rechitsky S, Verlinsky O, Tur-Kaspa I, Kalakoutis G, Angastiniotis M, Verlinsky Y. Preimplantation diagnosis and HLA typing for haemoglobin disorders. *Reprod Biomed Online* 2005; **11**: 362-370 [PMID: 16176679]

43 **Cotton F**, Lin C, Fontaine B, Gulbis B, Janssens J, Vertongen F. Evaluation of a capillary electrophoresis method for routine determination of hemoglobins A2 and F. *Clin Chem* 1999; **45**: 237-243 [PMID: 9931046]

44 **Mario N**, Baudin B, Aussel C, Giboudeau J. Capillary isoelectric focusing and high-performance cation-exchange chromatography compared for qualitative and quantitative analysis of hemoglobin variants. *Clin Chem* 1997; **43**: 2137-2142 [PMID: 9365399]

45 **Efremov CD**, Huisman TH, Bowman K, Wrightstone RN, Shroeder WA. Microchromatography of hemoglobins. II. A rapid method for the determination of hemoglobin A2. *J Lab Clin Med* 1974; **83**: 657-664 [PMID: 4817785]

46 **Galanello R**, Melis MA, Muroni P, Cao A. Quantitation of Hb a2 with DE-52 microchromatography in whole blood as screening test for beta-thalassemia heterozygotes. *Acta Haematol* 1977; **57**: 32-36 [PMID: 402762]

47 **Filla A**, De Michele G, Cavalcanti F, Pianese L, Monticelli A, Campanella G, Cocozza S. The relationship between trinucleotide (GAA) repeat length and clinical features in Friedreich ataxia. *Am J Hum Genet* 1996; **59**: 554-560 [PMID: 8751856]

48 **Ciotti P**, Di Maria E, Bellone E, Ajmar F, Mandich P. Triplet repeat primed PCR (TP PCR) in molecular diagnostic testing for Friedreich ataxia. *J Mol Diagn* 2004; **6**: 285-289 [PMID: 15507666]

49 **Puccio H**, Koenig M. Recent advances in the molecular pathogenesis of Friedreich ataxia. *Hum Mol Genet* 2000; **9**: 887-892 [PMID: 10767311]

50 **Angastiniotis M**, Vives Corrons JL, Soteriades ES, Eleftheriou A. The impact of migrations on the health services for rare diseases in Europe: the example of haemoglobin disorders. *ScientificWorldJournal* 2013; **2013**: 727905 [PMID: 23576907 DOI: 10.1155/2013/727905]

51 **Kontoghiorghes GJ**. A new era in iron chelation therapy: the design of optimal, individually adjusted iron chelation therapies for the complete removal of iron overload in thalassemia and other chronically transfused patients. *Hemoglobin* 2009; **33**: 332-338 [PMID: 19814679 DOI: 10.3109/03630260903217182]

52 **Mavrogeni SI**, Gotsis ED, Markussis V, Tsekos N, Politis C, Vretou E, Kermastinos D. T2 relaxation time study of iron overload in b-thalassemia. *MAGMA* 1998; **6**: 7-12 [PMID: 9794284]

53 **Anderson LJ**, Holden S, Davis B, Prescott E, Charrier CC, Bunce NH, Firmin DN, Wonke B, Porter J, Walker JM, Pennell DJ. Cardiovascular T2-star (T2*) magnetic resonance for the early diagnosis of myocardial iron overload. *Eur Heart J* 2001; **22**: 2171-2179 [PMID: 11913479]

54 **Kolnagou A**, Yazman D, Economides C, Eracleous E, Kontoghiorghes GJ. Uses and limitations of serum ferritin, magnetic resonance imaging T2 and T2* in the diagnosis of iron overload and in the ferrikinetics of normalization of the iron stores in thalassemia using the International Committee on

Chelation deferiprone/deferoxamine combination protocol. *Hemoglobin* 2009; **33**: 312-322 [PMID: 19814677 DOI: 10.3109/03630260903213231]
55 **Kolnagou A**, Fessas C, Papatryphonas A, Economides C, Kontoghiorghes GJ. Prophylactic use of deferiprone (L1) and magnetic resonance imaging T2* or T2 for preventing heart disease in thalassaemia. *Br J Haematol* 2004; **127**: 360-361; author reply 361-362 [PMID: 15491300]
56 **Kolnagou A**, Kontoghiorghes GJ. Future challenges in the use of magnetic resonance imaging for the diagnosis of iron overload. *Blood Transfus* 2010; **8**: 309-310 [PMID: 20967176 DOI: 10.2450/2010.0017-10]
57 **Di Bartolomeo P**, Santarone S, Di Bartolomeo E, Olioso P, Bavaro P, Papalinetti G, Di Carlo P, Papola F, Nicolucci A, Di Nicola M, Iacone A. Long-term results of survival in patients with thalassemia major treated with bone marrow transplantation. *Am J Hematol* 2008; **83**: 528-530 [PMID: 18383328 DOI: 10.1002/ajh.21175]
58 **Hongeng S**, Pakakasama S, Chuansumrit A, Sirachainan N, Sura T, Ungkanont A, Chuncharunee S, Jootar S, Issaragisil S. Reduced intensity stem cell transplantation for treatment of class 3 Lucarelli severe thalassemia patients. *Am J Hematol* 2007; **82**: 1095-1098 [PMID: 17674372]
59 **Issaragrisil S**, Visudhisakchai S, Suvatte V, Chandanayingyong D, Piankijagum A, Mahasandana C, Tanphaichitr VS. Bone marrow transplantation for thalassemia in Thailand. *Bone Marrow Transplant* 1993; **12** Suppl 1: 42-44 [PMID: 8374559]
60 **Andreani M**, Nesci S, Lucarelli G, Tonucci P, Rapa S, Angelucci E, Persini B, Agostinelli F, Donati M, Manna M. Long-term survival of ex-thalassemic patients with persistent mixed chimerism after bone marrow transplantation. *Bone Marrow Transplant* 2000; **25**: 401-404 [PMID: 10723583]
61 **Votsi C**, Zamba-Papanicolaou E, Middleton LT, Pantzaris M, Christodoulou K. A novel GBA2 gene missense mutation in spastic ataxia. *Ann Hum Genet* 2014; **78**: 13-22 [PMID: 24252062 DOI: 10.1111/ahg.12045]
62 **Kontoghiorghes GJ**, Kolnagou A. Molecular factors and mechanisms affecting iron and other metal excretion or absorption in health and disease: the role of natural and synthetic chelators. *Curr Med Chem* 2005; **12**: 2695-2709 [PMID: 16305466]
63 **Kontoghiorghes GJ**, Spyrou A, Kolnagou A. Iron chelation therapy in hereditary hemochromatosis and thalassemia intermedia: regulatory and non regulatory mechanisms of increased iron absorption. *Hemoglobin* 2010; **34**: 251-264 [PMID: 20524815 DOI: 10.3109/03630269.2010.486335]
64 **Kontoghiorghe CN**, Kolnagou A, Kontoghiorghes GJ. Potential clinical applications of chelating drugs in diseases targeting transferrin-bound iron and other metals. *Expert Opin Investig Drugs* 2013; **22**: 591-618 [PMID: 23586878 DOI: 10.1517/13543784.2013.787408]
65 **Hershko C**, Graham G, Bates GW, Rachmilewitz EA. Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol* 1978; **40**: 255-263 [PMID: 708645]
66 **al-Refaie FN**, Wickens DG, Wonke B, Kontoghiorghes GJ, Hoffbrand AV. Serum non-transferrin-bound iron in beta-thalassaemia major patients treated with desferrioxamine and L1. *Br J Haematol* 1992; **82**: 431-436 [PMID: 1419825]
67 **Kontoghiorghes GJ**, Goddard JG, Bartlett AN, Sheppard L. Pharmacokinetic studies in humans with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Clin Pharmacol Ther* 1990; **48**: 255-261 [PMID: 2401124]
68 **Kontoghiorghes GJ**. Iron chelation in biochemistry and medicine. In C Rice-Evans (Ed.). Free radicals, oxidant stress and drug action. Rechelieu press, London, 1987: 277-303
69 **Kontoghiorghes GJ**. Prospects for introducing deferiprone as potent pharmaceutical antioxidant. *Front Biosci* (Elite Ed) 2009; **1**: 161-178 [PMID: 19482634]
70 **Kontoghiorghes GJ**, Efstathiou A, Kleanthous M, Michaelides Y, Kolnagou A. Risk/benefit assessment, advantages over other drugs and targeting methods in the use of deferiprone as a pharmaceutical antioxidant in iron loading and non iron loading conditions. *Hemoglobin* 2009; **33**: 386-397 [PMID: 19814684 DOI: 10.3109/03630260903217141]
71 **Halliwell B**, Gutteridge JM. Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. *Hum Toxicol* 1988; **7**: 7-13 [PMID: 3278973]
72 **Richardson DR**, Lane DJ, Becker EM, Huang ML, Whitnall M, Suryo Rahmanto Y, Sheftel AD, Ponka P. Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol. *Proc Natl Acad Sci USA* 2010; **107**: 10775-10782 [PMID: 20495089 DOI: 10.1073/pnas.0912925107]
73 **Whitnall M**, Suryo Rahmanto Y, Huang ML, Saletta F, Lok HC, Gutiérrez L, Lázaro FJ, Fleming AJ, St Pierre TG, Mikhael MR, Ponka P, Richardson DR. Identification of nonferritin mitochondrial iron deposits in a mouse model of Friedreich ataxia. *Proc Natl Acad Sci USA* 2012; **109**: 20590-20595 [PMID: 23169664 DOI: 10.1073/pnas.1215349109]
74 **Lane DJ**, Huang ML, Ting S, Sivagurunathan S, Richardson DR. Biochemistry of cardiomyopathy in the mitochondrial disease Friedreich's ataxia. *Biochem J* 2013; **453**: 321-336 [PMID: 23849057 DOI: 10.1042/BJ20130079]
75 **Huang ML**, Becker EM, Whitnall M, Suryo Rahmanto Y, Ponka P, Richardson DR. Elucidation of the mechanism of mitochondrial iron loading in Friedreich's ataxia by analysis of a mouse mutant. *Proc Natl Acad Sci USA* 2009; **106**: 16381-16386 [PMID: 19805308 DOI: 10.1073/pnas.0906784106]
76 **Ramirez RL**, Qian J, Santambrogio P, Levi S, Koeppen AH. Relation of cytosolic iron excess to cardiomyopathy of Friedreich's ataxia. *Am J Cardiol* 2012; **110**: 1820-1827 [PMID: 23000103 DOI: 10.1016/j.amjcard.2012.08.018]
77 **Kontoghiorghes GJ**. Iron mobilization from ferritin using alpha-oxohydroxy heteroaromatic chelators. *Biochem J* 1986; **233**: 299-302 [PMID: 3954731]
78 **Kontoghiorghes GJ**, Chambers S, Hoffbrand AV. Comparative study of iron mobilization from haemosiderin, ferritin and iron(III) precipitates by chelators. *Biochem J* 1987; **241**: 87-92 [PMID: 3566714]
79 **Lodi R**, Cooper JM, Bradley JL, Manners D, Styles P, Taylor DJ, Schapira AH. Deficit of in vivo mitochondrial ATP production in patients with Friedreich ataxia. *Proc Natl Acad Sci USA* 1999; **96**: 11492-11495 [PMID: 10500204]
80 **Napier I**, Ponka P, Richardson DR. Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood* 2005; **105**: 1867-1874 [PMID: 15528311]
81 **Kontoghiorghes GJ**. Decrease solubilisation of ferritin iron and fresh iron (III) precipitate following repeated chelator treatments. *Inorg Chim Acta* 1987; **138**: 36-40 [DOI: 10.1016/S0020-1693(00)81178-0]
82 **Iancu TC**, Deugnier Y, Halliday JW, Powell LW, Brissot P. Ultrastructural sequences during liver iron overload in genetic hemochromatosis. *J Hepatol* 1997; **27**: 628-638 [PMID: 9365038]
83 **Iancu TC**, Neustein HB. Ferritin in human liver cells of homozygous beta-thalassaemia: ultrastructural observations. *Br J Haematol* 1977; **37**: 527-535 [PMID: 603778]
84 **Kolnagou A**, Michaelides Y, Kontos C, Kyriacou K, Kontoghiorghes GJ. Myocyte damage and loss of myofibers is the potential mechanism of iron overload toxicity in congestive cardiac failure in thalassemia. Complete reversal of the cardiomyopathy and normalization of iron load by deferiprone. *Hemoglobin* 2008; **32**: 17-28 [PMID: 18274979 DOI: 10.1080/03630260701726491]
85 **Lynch DR**, Deutsch EC, Wilson RB, Tennekoon G. Unanswered questions in Friedreich ataxia. *J Child Neurol* 2012; **27**: 1223-1229 [PMID: 22832776 DOI: 10.1177/0883073812453498]
86 **Kolnagou A**, Natsiopoulos K, Kleanthous M, Ioannou A,

Kontoghiorghes GJ. Liver iron and serum ferritin levels are misleading for estimating cardiac, pancreatic, splenic and total body iron load in thalassemia patients: factors influencing the heterogenic distribution of excess storage iron in organs as identified by MRI T2*. *Toxicol Mech Methods* 2013; **23**: 48-56 [PMID: 22943064 DOI: 10.3109/15376516.2012.727198]

87 **Kolnagou A**, Kleanthous M, Kontoghiorghes GJ. Efficacy, compliance and toxicity factors are affecting the rate of normalization of body iron stores in thalassemia patients using the deferiprone and deferoxamine combination therapy. *Hemoglobin* 2011; **35**: 186-198 [PMID: 21599431 DOI: 10.3109/03630269.2011.576153]

88 **Delatycki MB**, Camakaris J, Brooks H, Evans-Whipp T, Thorburn DR, Williamson R, Forrest SM. Direct evidence that mitochondrial iron accumulation occurs in Friedreich ataxia. *Ann Neurol* 1999; **45**: 673-675 [PMID: 10319894]

89 **Ashley CN**, Hoang KD, Lynch DR, Perlman SL, Maria BL. Childhood ataxia: clinical features, pathogenesis, key unanswered questions, and future directions. *J Child Neurol* 2012; **27**: 1095-1120 [PMID: 22859693 DOI: 10.1177/0883073812448840]

90 **Wilson RB**, Lynch DR, Fischbeck KH. Normal serum iron and ferritin concentrations in patients with Friedreich's ataxia. *Ann Neurol* 1998; **44**: 132-134 [PMID: 9667602]

91 **Waldvogel D**, van Gelderen P, Hallett M. Increased iron in the dentate nucleus of patients with Friedrich's ataxia. *Ann Neurol* 1999; **46**: 123-125 [PMID: 10401790]

92 **Michael S**, Petrocine SV, Qian J, Lamarche JB, Knutson MD, Garrick MD, Koeppen AH. Iron and iron-responsive proteins in the cardiomyopathy of Friedreich's ataxia. *Cerebellum* 2006; **5**: 257-267 [PMID: 17134988]

93 **Xia H**, Cao Y, Dai X, Marelja Z, Zhou D, Mo R, Al-Mahdawi S, Pook MA, Leimkühler S, Rouault TA, Li K. Novel frataxin isoforms may contribute to the pathological mechanism of Friedreich ataxia. *PLoS One* 2012; **7**: e47847 [PMID: 23082224 DOI: 10.1371/journal.pone.0047847]

94 **Greeley NR**, Regner S, Willi S, Lynch DR. Cross-sectional analysis of glucose metabolism in Friedreich ataxia. *J Neurol Sci* 2014; **342**: 29-35 [PMID: 24819921 DOI: 10.1016/j.jns.2014.04.015]

95 **Li MJ**, Peng SS, Lu MY, Chang HH, Yang YL, Jou ST, Lin DT, Lin KH. Diabetes mellitus in patients with thalassemia major. *Pediatr Blood Cancer* 2014; **61**: 20-24 [PMID: 24115521 DOI: 10.1002/pbc.24754]

96 **Platis O**, Anagnostopoulos G, Farmaki K, Posantzis M, Gotsis E, Tolis G. Glucose metabolism disorders improvement in patients with thalassaemia major after 24-36 months of intensive chelation therapy. *Pediatr Endocrinol Rev* 2004; **2** Suppl 2: 279-281 [PMID: 16462711]

97 **Selak MA**, Lyver E, Micklow E, Deutsch EC, Onder O, Selamoglu N, Yager C, Knight S, Carroll M, Daldal F, Dancis A, Lynch DR, Sarry JE. Blood cells from Friedreich ataxia patients harbor frataxin deficiency without a loss of mitochondrial function. *Mitochondrion* 2011; **11**: 342-350 [PMID: 21147271 DOI: 10.1016/j.mito.2010.12.003]

98 **Kontoghiorghes GJ**. The design of orally active iron chelators for the treatment of thalassaemia. PhD thesis. Colchester UK: University of Essex, British Library Microfilm No D66194/86, 1982: 1-243

99 **Kontoghiorghes GJ**. Design, properties, and effective use of the oral chelator L1 and other alpha-ketohydroxypyridines in the treatment of transfusional iron overload in thalassemia. *Ann N Y Acad Sci* 1990; **612**: 339-350 [PMID: 2291562]

100 **Kontoghiorghes GJ**, Pattichis K, Neocleous K, Kolnagou A. The design and development of deferiprone (L1) and other iron chelators for clinical use: targeting methods and application prospects. *Curr Med Chem* 2004; **11**: 2161-2183 [PMID: 15279556]

101 **Kontoghiorghes GJ**, Barr J, Nortey P, Sheppard L. Selection of a new generation of orally active alpha-ketohydroxypyridine iron chelators intended for use in the treatment of iron overload. *Am J Hematol* 1993; **42**: 340-349 [PMID: 8493983]

102 **Kontoghiorghes GJ**, Aldouri MA, Sheppard L, Hoffbrand AV. 1,2-Dimethyl-3-hydroxypyrid-4-one, an orally active chelator for treatment of iron overload. *Lancet* 1987; **1**: 1294-1295 [PMID: 2884415]

103 **Kontoghiorghes GJ**, Aldouri MA, Hoffbrand AV, Barr J, Wonke B, Kourouclaris T, Sheppard L. Effective chelation of iron in beta thalassaemia with the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Br Med J* (Clin Res Ed) 1987; **295**: 1509-1512 [PMID: 3122880]

104 **Vreugdenhil G**, Swaak AJ, Kontoghiorghes GJ, van Eijk HG. Efficacy and safety of oral iron chelator L1 in anaemic rheumatoid arthritis patients. *Lancet* 1989; **2**: 1398-1399 [PMID: 2574342]

105 **Agarwal MB**, Viswanathan C, Ramanathan J, Massil DE, Shah S, Gupte SS, Vasandani D, Puniyani RR. Oral iron chelation with L1. *Lancet* 1990; **335**: 601 [PMID: 1968588]

106 **Olivieri NF**, Templeton DM, Koren G, Chung D, Hermann C, Freedman MH, McClelland RA. Evaluation of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in iron-loaded patients. *Ann N Y Acad Sci* 1990; **612**: 369-377 [PMID: 2291564]

107 **Töndury P**, Kontoghiorghes GJ, Ridolfi-Lüthy A, Hirt A, Hoffbrand AV, Lottenbach AM, Sonderegger T, Wagner HP. L1 (1,2-dimethyl-3-hydroxypyrid-4-one) for oral iron chelation in patients with beta-thalassaemia major. *Br J Haematol* 1990; **76**: 550-553 [PMID: 2265118]

108 **Goudsmit R**. [Long-term treatment of patients with transfusion hemosiderosis using oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1)]. *Ned Tijdschr Geneeskd* 1991; **135**: 2133-2136 [PMID: 1944690]

109 **Kontoghiorghes GJ**. The proceedings of the 20th International Conference on Chelation held in the USA: advances on new and old chelation therapies. *Toxicol Mech Methods* 2013; **23**: 1-4 [PMID: 22900514 DOI: 10.3109/15376516.2012.720305]

110 **Kolnagou A**, Kontoghiorghes GJ. New golden era of chelation therapy in thalassaemia: the achievement and maintenance of normal range body iron stores. *Br J Haematol* 2010; **150**: 489-490; author reply 491 [PMID: 20507309 DOI: 10.1111/j.1365-2141.2010.08229]

111 **Kolnagou A**, Economides C, Eracleous E, Kontoghiorghes GJ. Long term comparative studies in thalassemia patients treated with deferoxamine or a deferoxamine/deferiprone combination. Identification of effective chelation therapy protocols. *Hemoglobin* 2008; **32**: 41-47 [PMID: 18274982 DOI: 10.1080/03630260701727085]

112 **Kolnagou A**, Kleanthous M, Kontoghiorghes GJ. Reduction of body iron stores to normal range levels in thalassaemia by using a deferiprone/deferoxamine combination and their maintenance thereafter by deferiprone monotherapy. *Eur J Haematol* 2010; **85**: 430-438 [PMID: 20662901 DOI: 10.1111/j.1600-0609.2010.01499.x]

113 **Farmaki K**, Tzoumari I, Pappa C, Chouliaras G, Berdoukas V. Normalisation of total body iron load with very intensive combined chelation reverses cardiac and endocrine complications of thalassaemia major. *Br J Haematol* 2010; **148**: 466-475 [PMID: 19912219 DOI: 10.1111/j.1365-2141.2009.07970.x]

114 **Kolnagou A**, Kontoghiorghes GJ. Maintenance of normal range body iron store levels for up to 4.5 years in thalassemia major patients using deferiprone monotherapy. *Hemoglobin* 2010; **34**: 204-209 [PMID: 20524810 DOI: 10.3109/03630269.2010.485890]

115 **Wood JC**, Kang BP, Thompson A, Giardina P, Harmatz P, Glynos T, Paley C, Coates TD. The effect of deferasirox on cardiac iron in thalassemia major: impact of total body iron stores. *Blood* 2010; **116**: 537-543 [PMID: 20421452 DOI: 10.1182/blood-2009-11-250308]

116 **Tanner MA**, Galanello R, Dessi C, Smith GC, Westwood MA, Agus A, Pibiri M, Nair SV, Walker JM, Pennell DJ.

Combined chelation therapy in thalassemia major for the treatment of severe myocardial siderosis with left ventricular dysfunction. *J Cardiovasc Magn Reson* 2008; **10**: 12 [PMID: 18298856 DOI: 10.1186/1532-429X-10-12]

117 **Rajapurkar MM**, Hegde U, Bhattacharya A, Alam MG, Shah SV. Effect of deferiprone, an oral iron chelator, in diabetic and non-diabetic glomerular disease. *Toxicol Mech Methods* 2013; **23**: 5-10 [PMID: 22978744 DOI: 10.3109/15376516.2012.730558]

118 **Boddaert N**, Le Quan Sang KH, Rötig A, Leroy-Willig A, Gallet S, Brunelle F, Sidi D, Thalabard JC, Munnich A, Cabantchik ZI. Selective iron chelation in Friedreich ataxia: biologic and clinical implications. *Blood* 2007; **110**: 401-408 [PMID: 17379741]

119 **Abbruzzese G**, Cossu G, Balocco M, Marchese R, Murgia D, Melis M, Galanello R, Barella S, Matta G, Ruffinengo U, Bonuccelli U, Forni GL. A pilot trial of deferiprone for neurodegeneration with brain iron accumulation. *Haematologica* 2011; **96**: 1708-1711 [PMID: 21791473 DOI: 10.3324/haematol.2011.043018]

120 **Devos D**, Moreau C, Devedjian JC, Kluza J, Petrault M, Laloux C, Jonneaux A, Ryckewaert G, Garçon G, Rouaix N, Duhamel A, Jissendi P, Dujardin K, Auger F, Ravasi L, Hopes L, Grolez G, Firdaus W, Sablonnière B, Strubi-Vuillaume I, Zahr N, Destée A, Corvol JC, Pöltl D, Leist M, Rose C, Defebvre L, Marchetti P, Cabantchik ZI, Bordet R. Targeting chelatable iron as a therapeutic modality in Parkinson's disease. *Antioxid Redox Signal* 2014; **21**: 195-210 [PMID: 24251381]

121 **Myers L**, Farmer JM, Wilson RB, Friedman L, Tsou A, Perlman SL, Subramony SH, Gomez CM, Ashizawa T, Wilmot GR, Mathews KD, Balcer LJ, Lynch DR. Antioxidant use in Friedreich ataxia. *J Neurol Sci* 2008; **267**: 174-176 [PMID: 17988688]

122 **Kearney M**, Orrell RW, Fahey M, Pandolfo M. Antioxidants and other pharmacological treatments for Friedreich ataxia. *Cochrane Database Syst Rev* 2012; **4**: CD007791 [PMID: 22513953 DOI: 10.1002/14651858.CD007791.pub3]

123 **Kontoghiorghes GJ**. Iron mobilization from transferrin and non-transferrin-bound-iron by deferiprone. Implications in the treatment of thalassemia, anemia of chronic disease, cancer and other conditions. *Hemoglobin* 2006; **30**: 183-200 [PMID: 16798643]

124 **Evans RW**, Sharma M, Ogwang W, Patel KJ, Bartlett AN, Kontoghiorghes GJ. The effect of α-ketohydroxypyridine chelators on transferrin saturation in vitro and in vivo. *Drugs Today* 1992; **28** (Suppl A): 9-23

125 **Sheppard L**, Kontoghiorghes GJ. Synthesis and metabolism of L1 and other novel alpha-ketohydroxypyridine iron chelators and their metal complexes. *Drugs Today* 1992; **28** (Suppl A): 3-10

126 **Kontoghiorghes GJ**, Bartlett AN, Hoffbrand AV, Goddard JG, Sheppard L, Barr J, Nortey P. Long-term trial with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1). I. Iron chelation and metabolic studies. *Br J Haematol* 1990; **76**: 295-300 [PMID: 2094333]

127 **Kontoghiorghes GJ**, Kolnagou A, Peng CT, Shah SV, Aessopos A. Safety issues of iron chelation therapy in patients with normal range iron stores including thalassaemia, neurodegenerative, renal and infectious diseases. *Expert Opin Drug Saf* 2010; **9**: 201-206 [PMID: 20059374 DOI: 10.1517/14740330903535845]

128 **Kakhlon O**, Manning H, Breuer W, Melamed-Book N, Lu C, Cortopassi G, Munnich A, Cabantchik ZI. Cell functions impaired by frataxin deficiency are restored by drug-mediated iron relocation. *Blood* 2008; **112**: 5219-5227 [PMID: 18796625 DOI: 10.1182/blood-2008-06-161919]

129 **Velasco-Sánchez D**, Aracil A, Montero R, Mas A, Jiménez L, O'Callaghan M, Tondo M, Capdevila A, Blanch J, Artuch R, Pineda M. Combined therapy with idebenone and deferiprone in patients with Friedreich's ataxia. *Cerebellum* 2011; **10**: 1-8 [PMID: 20865357 DOI: 10.1007/s12311-010-0212-7]

130 **Arpa J**, Sanz-Gallego I, Rodríguez-de-Rivera FJ, Domínguez-Melcón FJ, Prefasi D, Oliva-Navarro J, Moreno-Yangüela M. Triple therapy with deferiprone, idebenone and riboflavin in Friedreich's ataxia - open-label trial. *Acta Neurol Scand* 2014; **129**: 32-40 [PMID: 23668357 DOI: 10.1111/ane.12141]

131 **Cossu G**, Abbruzzese G, Matta G, Murgia D, Melis M, Ricchi V, Galanello R, Barella S, Origa R, Balocco M, Pelosin E, Marchese R, Ruffinengo U, Forni GL. Efficacy and safety of deferiprone for the treatment of pantothenate kinase-associated neurodegeneration (PKAN) and neurodegeneration with brain iron accumulation (NBIA): results from a four years follow-up. *Parkinsonism Relat Disord* 2014; **20**: 651-654 [PMID: 24661465 DOI: 10.1016/j.parkreldis.2014.03.002]

132 **Kolnagou A**, Economides C, Eracleous E, Kontoghiorghes GJ. Low serum ferritin levels are misleading for detecting cardiac iron overload and increase the risk of cardiomyopathy in thalassemia patients. The importance of cardiac iron overload monitoring using magnetic resonance imaging T2 and T2*. *Hemoglobin* 2006; **30**: 219-227 [PMID: 16798647]

133 **Hayashi S**, Peranteau WH, Shaaban AF, Flake AW. Complete allogeneic hematopoietic chimerism achieved by a combined strategy of in utero hematopoietic stem cell transplantation and postnatal donor lymphocyte infusion. *Blood* 2002; **100**: 804-812 [PMID: 12130490]

134 **Skordis N**, Petrikkos L, Toumba M, Hadjigavriel M, Sitarou M, Kolnakou A, Skordos G, Pangalou E, Christou S. Update on fertility in thalassaemia major. *Pediatr Endocrinol Rev* 2004; **2** Suppl 2: 296-302 [PMID: 16462715]

135 **Vreugdenhil G**, Kontoghiorghes GJ, Van Eijk HG, Swaak AJ. Impaired erythropoietin responsiveness to the anaemia in rheumatoid arthritis. A possible inverse relationship with iron stores and effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Clin Exp Rheumatol* 1991; **9**: 35-40 [PMID: 2054965]

136 **Mohanty D**, Ghosh K, Pathare AV, Karnad D. Deferiprone (L1) as an adjuvant therapy for Plasmodium falciparum malaria. *Indian J Med Res* 2002; **115**: 17-21 [PMID: 12424933]

137 **Pandolfo M**, Hausmann L. Deferiprone for the treatment of Friedreich's ataxia. *J Neurochem* 2013; **126** Suppl 1: 142-146 [PMID: 23859349 DOI: 10.1111/jnc.12300]

138 **Koeppen AH**, Ramirez RL, Yu D, Collins SE, Qian J, Parsons PJ, Yang KX, Chen Z, Mazurkiewicz JE, Feustel PJ. Friedreich's ataxia causes redistribution of iron, copper, and zinc in the dentate nucleus. *Cerebellum* 2012; **11**: 845-860 [PMID: 22562713 DOI: 10.1007/s12311-012-0383-5]

139 **Adhemar JP**, Laederich J, Jaudon MC, Masselot JP, Galli A, Kleinknecht D. Removal of aluminium from patients with dialysis encephalopathy. *Lancet* 1980; **2**: 1311 [PMID: 6108489]

140 **Wu Z**, Du Y, Xue H, Wu Y, Zhou B. Aluminum induces neurodegeneration and its toxicity arises from increased iron accumulation and reactive oxygen species (ROS) production. *Neurobiol Aging* 2012; **33**: 199.e1-199.12 [PMID: 20674094 DOI: 10.1016/j.neurobiolaging.2010.06.018]

141 **Weigel KJ**, Lynch SG, Levine SM. Iron chelation and multiple sclerosis. *ASN Neuro* 2014; **6**: e00136 [PMID: 24397846 DOI: 10.1042/AN20130037]

142 **Kontoghiorghes GJ**. Comparative efficacy and toxicity of desferrioxamine, deferiprone and other iron and aluminium chelating drugs. *Toxicol Lett* 1995; **80**: 1-18 [PMID: 7482575]

143 **Kontoghiorghes GJ**, Barr J, Baillod RA. Studies of aluminium mobilization in renal dialysis patients using the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one. *Arzneimittelforschung* 1994; **44**: 522-526 [PMID: 8011008]

144 **Pashalidis I**, Kontoghiorghes GJ. Competition studies of L1-deferiprone with copper and iron. Possible implications on efficacy, toxicity and new therapeutic applications. *Transfus Sci* 2000; **23**: 259-261 [PMID: 11099910]

145 **Efrati S**, Ben-Jacob E. Reflections on the neurotherapeutic

effects of hyperbaric oxygen. *Expert Rev Neurother* 2014; **14**: 233-236 [PMID: 24471697 DOI: 10.1586/14737175.2014.884928]
146 **Evans-Galea MV**, Pébay A, Dottori M, Corben LA, Ong SH, Lockhart PJ, Delatycki MB. Cell and gene therapy for Friedreich ataxia: progress to date. *Hum Gene Ther* 2014; **25**: 684-693 [PMID: 24749505]
147 **Drakopoulou E**, Papanikolaou E, Georgomanoli M, Anagnou NP. Towards more successful gene therapy clinical trials for β-thalassemia. *Curr Mol Med* 2013; **13**: 1314-1330 [PMID: 23865429]

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ORIGINAL ARTICLE

Methodical and pre-analytical characteristics of a multiplex cancer biomarker immunoassay

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Abstract

AIM: To test the methodical and pre-analytical performance of a new multiplex cancer biomarker panel using magnetic beads.

METHODS: The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 comprises the tumor markers carcinoembryonic antigen, alpha-fetoprotein, total prostate-specific antigen, cancer antigen 15-3, cancer antigen 19-9, cancer antigen 125, cytokeratine 19-fragment, β-human chorionic gonadotropin, human epididymis protein 4, osteopontin, prolactin, the cell death and angiogenesis markers soluble Fas, soluble Fas-ligand, tumor necrosis factor related apoptosis-inducing ligand, vascular endothelial growth factor and the immunological markers interleukin-6 (IL-6), IL-8, tumor necrosis factor-α, transforming growth factor α, fibroblast growth factor-2, macrophage migration inhibitory factor, leptin, hepatocyte growth factor, and stem cell factor. We determined intra- and inter-assay imprecision as well as dilution linearity using quality controls and serum pools. Furthermore, the stability of the 24 biomarkers examined in this panel was ascertained by testing the influence of different storage temperatures and time span before centrifugation.

RESULTS: For all markers measured in the synthetic internal quality controls, the intra-assay imprecision ranged between 2.26% and 9.41%, while for 20 of 24 measured markers in the physiological serum pools, it ranged between 1.68% and 12.87%. The inter-assay imprecision ranged between 1.48%-17.12% for 23 biomarkers in synthetic, and between 4.59%-23.88% for 18 biomarkers in physiological quality controls. Here, single markers with very low concentration levels had increased imprecision rates. Dilution linearity was acceptable (70%-130% recovery) for 20 biomarkers. Regarding pre-analytical influencing factors, most markers were stable if blood centrifugation was delayed or if serum was stored for up to 24 h at 4 ℃ and 25 ℃ after centrifugation. Comparable results were obtained in serum and plasma for most markers. However, great changes were observed for single markers.

CONCLUSION: MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 assay is a stable and precise method for detection of most biomarkers included in the kit. However, single markers have to be interpreted with care.

Key words: Multiplex immunoassay; Tumor marker; Cytokines; Cell death markers; Methodical evaluation

Core tip: In this study, the methodological quality of a new research-use-only multiplex magnetic bead assay,

particularly designed for cancer diagnosis, was evaluated. This attractive panel includes 24 biomarkers: established as well as auspicious tumor markers and markers deriving from the fields of apoptosis, immunology and angiogenesis. Herewith, the complexity and multifactorial background of a cancer disease is depicted. Measurements were performed with physiological serum pools and intra- and inter-assay imprecision as well as dilution linearity were assessed. Furthermore, the influence of preanalytical factors was investigated.

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INTRODUCTION

Despite of essential achievements in cancer research concerning diagnosis, therapy options and follow up methods, cancer diseases still present a global health problem[1]. A great variety of clinical and imaging tools are applied to diagnose tumor masses and screening programs have been established for certain entities[2]. Some serum tumor markers, such as alpha-fetoprotein (AFP), cancer antigen 125 (CA 125), CA 15-3, CA 19-9, carcinoembryonic antigen (CEA) or prostate-specific antigen (PSA), have been introduced as supplementary diagnostic tools, but none of the above is recommended as a singular method to define a cancer diagnosis[3-5].

Cancer is nowadays perceived as a complex disease involving inflammatory and immunological systems and programs of cell death[6,7]. Thus, the diagnostic opportunity could be greatly enhanced by measurement of more than one marker as a fraction of information required to understand a complex pathological state[8,9]. Based on these findings, methods for parallel tumor marker testing have become more and more interesting in cancer research. Here, biomarkers, representing different systemic processes, such as inflammation, angiogenesis or cell death, can be combined with established tumor markers in one panel and potentially increase diagnostic accuracy[10-12].

Multiplex based immunoassays belong to the leading methods in this field. They are based on flow cytometry principles applied to labeled microspheres and depict an "ELISA on a bead"[13]. They offer several advantages, such as high-throughput performance, low material requirement, wide range application and cost- and time-effective multiplexing of more than 20 parameters[8,13].

However, the implementation of bead based multiplex assays has not yet been established in clinical routine[14]. Currently used tumor markers are mainly tested with single parameter assays. Not least due to the great potential of differently composed assays or marker panels, respectively, this field requires further research in order to assess assay quality, increase comparability of multiplex assays, and to encourage consistent guidelines which as of yet are non-existent[3,9,15].

As already shown by other research groups marker combination has the potential to greatly improve the quality of early diagnosis and other therapeutically relevant applications[12,16,17]. Several manufacturers offer diverse panels of markers, mainly for the combined measurement of many immunological and metabolic markers. For oncological purposes, the MILLIPLEX® Map Human Circulating Cancer Biomarker Magnetic Bead Panel Kit (EMD Millipore) was recently released. It represents an attractive option particularly for study settings. This kit includes reagents for the detection of 24 biomarkers, which portray a widespread spectrum of already validated as well as upcoming auspicious oncological, cell death, angiogenesis and immunological biomarkers, such as CEA, AFP, total prostate-specific antigen (total-PSA), CA 15-3, CA 19-9, CA 125, cytokeratine 19-fragment (CYFRA 21-1), β-human chorionic gonadotropin (β-HCG), human epididymis protein 4 (HE4), osteopontin (OPN), prolactin, soluble Fas (sFas), soluble Fas-ligand (sFasL), tumor necrosis factor related apoptosis-inducing ligand (TRAIL), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), IL-8, tumor necrosis factor-α (TNFα), transforming growth factor α (TGFα), fibroblast growth factor-2 (FGF2), macrophage migration inhibitory factor (MIF), leptin, hepatocyte growth factor (HGF) and stem cell factor (SCF).

However, in order to be used in studies and for clinical measurements, this panel must fulfill certain requirements, such as high reliability, accuracy, robustness as well as high analytical and clinical sensitivity and specificity[18,19]. Furthermore, the analytes must be stable against potentially influencing pre-analytical factors[3]. This study was carried out to critically test whether all or only some of the markers fulfill these basic methodical quality criteria and can thus be recommended for application in clinical or study conditions.

MATERIALS AND METHODS

In order to assess the methodological performance of MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, 96 Well Plate Assay we tested intra- and inter-assay imprecision as well as dilution linearity.

Standard samples plus quality controls QC 1 and QC 2 delivered by the kits were used for the internal methodological control. Standard 7 depicted the basis for a dilution line with the factor 1:3 from high to lower biomarker concentrations. The standard dilution line as well as the concentrations of QC 1 and QC 2 were predefined by the manufacturer.

For external control, we produced two serum pools with levels in the moderate to high and in the very low value range for most markers (pool 1 and pool 2). To create pool 1, 37 residual and anonymized sera of daily

clinical routine diagnostics were combined. Inclusion criteria were present high levels of the inflammation parameter C-reactive protein and well above average levels of the biomarkers AFP, β-HCG, CA 15-3, CA 125, CA 19-9, CEA, NSE and PSA. Here, patient history was not considered. Pool 2 is a combination of two sera taken from young healthy women (mean age 23.5 years). The sera pools, standard samples and quality controls QC 1 and QC 2 were run in duplicate as minimum within each plate.

In order to evaluate the linearity of dilution, a 50% dilution of the higher concentrated pool 1 was prepared by mixing the pool 1 sample with the appropriate amount of serum matrix enclosed in our kit. We defined the acceptable range for the recoveries as values between 70% and 130%.

Next, the estimation of possible affecting pre-analytical issues was tested. Briefly, samples of two different patients were stored at 25 ℃ (room temperature) for 6 and 24 h prior to centrifugation and subsequent freezing at -80 ℃. In a further experiment, samples were stored at 4 ℃ and 25 ℃ for 6, 24 and 48 h, respectively, after centrifugation and before freezing at -80 ℃. As reference control we used the corresponding samples, which were directly frozen after centrifugation. Finally, biomarkers were tested in serum and EDTA-plasma samples that were taken in parallel from the two healthy donors. All different conditions were measured in a single plate at a later time point to avoid inter-assay interferences.

MILLIPLEX® MAP Kit on the Bio-Plex® 200 System

The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, 96 well plate assay purchased from EMD Millipore included all the reagents as well as an appropriate plate required by the assay. The procedure was conducted by experienced staff according to the manufacturer's protocol. For washing steps, the Bio-Plex® Pro Ⅱ wash station was applied. All plates were run on the Bio-Plex® 200 System. Before each assay run, the system was calibrated with the Bio-Plex® calibration kit and validated with the Bio-Plex® validation kit 4.0. Bio-Plex® sheath fluid served as the delivery medium for the samples. Analysis was performed with Bio-Plex® manager 6.1. Within the device settings, 50 events per bead region were defined as minimum criterion.

Principle

MILLIPLEX® MAP Kit Human Circulating Cancer Biomarker Magnetic Bead Panel 1 was developed as an immunoassay on the surface of fluorescent-coded magnetic beads (MagPlex™-C microspheres). The proportion of two fluorescent dyes on these beads forms the code and determines in such way up to 100 different kinds of beads. Here, we have 24 differently coded bead groups, each of which is coated with a specific capture antibody to detect one of the 24 biomarkers which are CEA, AFP, PSA, CA 15-3, CA 19-9, CA 125, CYFRA 21-1, β-HCG, HE4, osteopontin and prolactin, the cell death and angiogenesis markers sFas, sFasL, TRAIL and VEGF as well as the immunological markers IL-6, IL-8, TNFα, TGFα, FGF-2, MIF, leptin, HGF and SCF.

The binding of specific analytes begins in the bead mixture suspended with a test sample. Next, a biotinylated detection antibody is introduced and subsequent incubation with streptavidin-phycoerythrin (PE) conjugate is performed to complete the reaction on the microspheres.

Finally, the assay is analyzed by the Bio-Plex® 200 system. Here, the beads coupled with the capture antibody bound to the specific analyte, biotinylated detection antibody and streptavidin-PE on its surfaces pass through a laser, which excites the internal dyes. A second laser excites the signal of PE. High-speed digital-signal processors identify the beads and detect the fluorescent signal intensity in order to quantify the assay result.

Procedures

All reagents and sera were brought to room temperature before use. Wash buffer, assay buffer, serum matrix, standard 7, quality controls 1 and 2, beads, detection antibodies, and streptavidin-PE were prepared as recommended by the manufacturer. Serum samples were thawed and individually vortexed for 15 s. Thereafter, they were centrifuged (Eppendorf centrifuge 581OR) at 3500 rpm for one min. Next, 15 μL of sample was mixed with 75 μL of serum matrix creating a 1:6 dilution. To create a homogeneous mixture of all antibody conjugated beads, every vial containing one set of microspheres was sonicated for 30 s and then vortexed for one minute. One hundred and fifty microliter of each vial was transferred into a mixing bottle that was vortexed again for one minute. The beads were protected from exposure to light throughout the assay. For pre-wetting, 200 μL assay buffer was pipetted into each well, the plate was covered with a sealer and then shaken at 700 rpm for 10 min. The fluid was removed by tapping the plate on a paper towel and centrifuging it briefly at 3500 rpm lying top down on a paper sheet in the centrifuge. Twenty-five microliter of background, standard 1-7 and quality controls 1 and 2 were pipetted in duplicate into the appropriate wells and 25 μL of serum matrix was added. Next, sample wells were filled with 25 μL of assay buffer and 25 μL of the diluted sera was pipetted into the appropriate wells. Finally, the magnetic bead mixture was vortexed for 1 min and 25 μL were pipetted into each well. The plate was sealed, covered with aluminum foil and then shaken at 700 rpm for 16 h at 4 ℃. After incubation time the magnetic bead plates were washed as recommended by the method protocol three times using assay buffer by means of Bio-Plex® Pro Ⅱ wash station. Then 25 μL of detection antibodies were added to each well, the plate was sealed and covered with aluminum foil and shaken at 700 rpm for 1 h. Now 25 μL of streptavidin-PE per well were added and the plate was again sealed, covered with aluminum foil and shaken at 700 rpm for 30 min. Thereon the three washing steps were performed as described above. One hundred microliter of sheath fluid

were pipetted into each well, the plate sealed and covered with aluminum foil and shaken at 700 rpm for 5 min in order to resuspend the beads. Lastly, the plates were run on the Bio-Plex® 200 system.

Expected concentrations of each tested biomarker for standards 1-7 were entered into the system prior to running the assay. The device detects appropriate fluorescence intensities (FI) and creates a standard curve for each marker. These curves are generated by linking the measured FI values with the expected concentration of markers in standards 1-7. Further translation of FI-values in concentration levels of all the following samples is based on these curves. Depending on the five parameter logistic, the function possesses predefined points of accepted extrapolation, which are the minimum and maximum asymptotes. For the lower limits in our study, we accepted an extrapolation in round terms in the middle between the lowest standard and the minimum asymptote point. Due to the phenomenon of heteroscedasticity towards higher concentrations, here, the accepted extrapolation was defined as an approximation of the highest standard value. These limits consequently illustrate our measuring range.

As all physiological serum samples were diluted 1:6 with serum matrix included into the kit, the dilution factor 6 was considered by the software before yielding the final concentration of the samples. For convenience of comparability in added tables, we multiplied all non diluted concentrations (accepted measuring range, observed concentration of QC 1, QC 2 and standard 5) with the factor 6.

The results from the software of Bio-Plex® 200 contain the measured FI and when duplicates were run, also the corresponding means, standard deviation and coefficients of variation (CV in %) as well as the corresponding concentrations.

Statistical analysis

In order to assess intra- and inter-assay imprecision FI-based CVs were used. We also determined the CVs based on the observed concentration for the analysis. Means and ranges were calculated for all comparisons. In order to quantify the dilution linearity, we determined observed concentration-based recoveries related to the corresponding expected values for the 50% dilution of pool 1. The evaluation of the pre-analytical influence of different storage conditions is represented by calculated recoveries based on FI results.

All in all, we tested ten kits or rather ten plates. The first five plates were ordered as a batch and were measured subsequently and strictly under the same conditions. The following five plates were of the same lot, but were ordered and measured about six months later under different conditions. Therefore, the main method evaluation in our study is based on the first five kits. However, results of the overall evaluation are also shown. In one assay, a pipetting error of the pool samples occurred and the respective values were omitted.

RESULTS

Intra-assay imprecision

The intra-assay imprecision as the mean CV (in %) over all plates for synthetic quality controls QC 1, QC 2 and standard 5 as well as for physiological serum pools 1 and 2 was calculated for five different magnetic plates relating to each tested biomarker. Here, only results within the measuring range were included.

In QC 1 and QC 2, 22 and 24 markers had an FI-based CV below 10%. In QC 2, 21 biomarkers were measured with a CV less than 5%. The CVs ranged between 3.81% (AFP) and 13.38% (FGF2) for QC 1 and between 2.06% (total PSA) and 5.56% (β-HCG) for QC 2. Observed concentration-based CVs ranged between 4.23% (AFP) and 9.41% (FGF2) for QC 1 and between 2.26% (TRAIL) and 7.69% (CA 19-9) for QC 2 (Table 1).

In the standard 5-sample, all of the 24 biomarkers were measured with CV values below 5%. The range was between 0.91% (OPN)-4.41% (VEGF).

In the physiological serum pool 1, all biomarkers showed an FI-based CV below 10%, while 13 biomarkers had a CV below 5%. The values ranged from 1.89% (MIF) to 8.71% (FGF2). Observed concentration-based CVs ranged from 1.68% (MIF) to 36.09% (β-HCG) with 12 biomarkers measured with a CV below 5% and four biomarkers exceeding the 10% range (Table 1).

In physiological serum pool 2, FI-based imprecision ranged from 1.65% (CA 19-9) to 14.31% (MIF) with only one CV (MIF) found to be higher than 10%. CVs of 11 biomarkers fell below 5%. Observed concentration-based imprecision ranged from 1.47% (sFas)-15.66% (MIF) with seven biomarkers measured with a CV below 5% and seven biomarkers exceeding the 10% range (Table 1).

When intra-assay imprecision was evaluated for all ten plates, the CVs were somewhat higher for pools and QC samples (Table 2).

Inter-assay imprecision

FI-based imprecision: The inter-assay imprecision was performed by calculating the CV in % involving all fluorescence intensity results for QC 1, QC 2, standard 5, pool 1 and pool 2 over five different magnetic plates relating to each tested marker. Here, only results within the measuring range were included.

For synthetic QC 1, QC 2 and standard 5, 16, 17 and 18 markers had imprecision below 20%, ranging between 8.85% (IL-8) and 45.75% (OPN), 8.88% (CEA) and 29.04% (OPN) as well as between 8.61% (total PSA) and 42.71% (CYFRA 21-1) (Table 3).

In the higher-concentrated physiological serum pool 1, the inter-assay imprecision fell below 20% for 18 biomarkers with the total range between 4.49% (β-HCG) and 46.72% (OPN). In the very low serum pool 2, only four biomarkers were measured with a CV below 20%, collectively ranging between 10.19% (HGF) and 85.54% (OPN) (Table 3).

Table 1 Intra-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2505	7.24	8.87	10933	2.32	3.39	2150	1.29		38897	2.29	3.98	466	6.77	7.78
AFP	500-600000	pg/mL	11980	3.81	4.23	54865	2.4	3.29	10703	2.88		455084	3.46	12.87	1598	4.99	11.99
Total PSA	50-60000	pg/mL	1230	5.97	7.9	5880	2.06	3.41	1057	1.92		7762	2.46	4.16	< MR	< MR	< MR
CA 15-3	0.5-600	U/mL	12	9.1	8.43	57	4.06	4.5	11	3.05		29	6.48	6.35	24	3.87	3.7
CA 19-9	2-3000	U/mL	61	9.54	7.35	292	5.27	7.69	54	3.2		33	3.35	2.66	10	1.65	1.75
CA 125	2-4000	U/mL	71	6.77	6.68	347	4.14	4.4	68	2.27		302	4.15	4.36	5.53	6.23	11.25
β-HCG	0.2-400	mU/mL	8	8.86	5.54	39	5.56	4.25	7	1.54		0.65	6.37	36.09	< MR	< MR	< MR
CYFRA 21-1	500-900000	pg/mL	29454	7.92	6.98	155151	3.7	3.18	16614	1.52		9724	4.22	4.77	3506	8.07	13.12
HE4	2000-3000000	pg/mL	55648	8.73	6.98	291745	5.17	5.23	55123	4.1		2276	8.16	9.58	< MR	< MR	< MR
Prolactin	500-600000	pg/mL	11868	5.69	4.73	60195	3.04	3.55	10922	2.61		12057	4.37	3.69	10765	3.08	2.52
Leptin	500-600000	pg/mL	13056	8.97	5.05	62955	2.75	2.38	10971	1.94		13430	7.17	4.04	8468	2.78	1.48
OPN	2000-3000000	pg/mL	53895	8.94	7.32	236975	3.72	6.02	43171	0.91		73954	4.46	4.01	10870	2.75	2.25
HGF	100-120000	pg/mL	2452	9.76	8.64	12766	4.06	4.28	2154	2.86		785	3.11	2.86	283	5.65	7.44
MIF	100-120000	pg/mL	2230	7.83	5.97	11061	4.5	4.45	2209	3.06		574	1.89	1.68	513	14.31	15.66
sFas	100-150000	pg/mL	2486	6.3	5.53	12705	3.19	2.77	2779	2.4		3548	4.52	3.89	2217	1.67	1.47
sFasL	50-60000	pg/mL	1219	5.65	5.71	5898	2.17	2.28	1091	2.86		< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	252	7.23	7.41	1233	2.16	2.26	221	2.49		65	3.4	3.62	92	3.32	3.5
VEGF	50-60000	pg/mL	1127	13.04	8.8	6110	3.97	2.78	1115	4.41		< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	49	9.2	9.27	226	4.33	4.21	53	3.43		16	5.66	7.02	< MR	< MR	< MR
IL-8	5-6000	pg/mL	122	5.53	5.67	605	2.19	2.34	110	1.71		33	5.85	6.7	6.71	4.49	9.43
TNFα	5-6000	pg/mL	114	7.77	7.97	562	2.88	3.17	110	2.25		13	4.56	5.66	6.41	3.12	6.68
TGFα	10-12000	pg/mL	237	6.36	6.29	1180	3.02	3.11	221	1.23		24	5.17	8.75	18	4.49	11.59
FGF2	50-50000	pg/mL	1101	13.38	9.41	5571	2.61	2.85	1066	3.08		55	8.71	18.11	79	9.57	14.52
SCF	20-30000	pg/mL	599	8.41	7.97	2813	3.02	2.88	550	1.17		58	6.99	10.97	50	8.32	13.4

Intra-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of assays 1-5 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

Observed concentration based imprecision: In synthetic internal controls, the imprecision was below 20% for 23 biomarkers in QC 1 and QC 2. The same applied to 24 biomarkers in standard 5. The corresponding ranges were 2.44% (Leptin) to 27.37% (CYFRA 21-1), 1.48% (β-HCG) to 40.96% (CYFRA 21-1) and 0.32% (total PSA) to 4.15% (CEA) (Table 3).

In the physiological serum pool 1, the imprecision fell below 20% for 16 biomarkers and values ranged from 4.59% (total PSA) to 68.81% (FGF2). Notably, single markers in pool 1 with very low concentration levels had considerably higher imprecision rates. In serum pool 2 with very low values for all markers, only one marker (CEA) was measured with a CV less than 20%. Here, the imprecision ranged in total between 14.43% (CEA)-66.04% (AFP) (Table 3).

When inter-assay imprecision was evaluated for all ten plates, the CVs were somewhat higher for pools and QC samples (Table 4).

Table 2 Extended intra-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2545	8.32	11.34	12727	7.61	12.23	2146	4.11		39391	2.1	3.63	560	8.09	5.67
AFP	500-600000	pg/mL	12038	6.09	6.9	56960	3.08	4.23	10754	4.45		493993	2.39	8.73	1489	6.87	11.8
Total PSA	50-60000	pg/mL	1421	5.76	7.78	6172	3.75	6.27	1052	4.1		8246	1.59	2.69	68	9.76	7.02
CA 15-3	0.5-600	U/mL	12	9	8.7	62	4.18	4.96	11	4.32		33	5.31	5.23	26	9.19	8.66
CA 19-9	2-3000	U/mL	64	10.63	8.63	329	4.83	7.1	54	5.05		36	2.9	2.39	10	4.87	6.17
CA 125	2-4000	U/mL	74	7.02	7.03	370	3.61	3.78	68	2.84		317	3.59	3.64	6.13	4.74	8.54
β-HCG	0.2-400	mU/mL	7.85	10.62	7.02	42	4.89	3.67	7	5.08		1.2	6.76	18.28	0.55	8.17	31.39
CYFRA 21-1	500-900000	pg/mL	24985	7.62	8.22	152731	12.13	4.28	16601	3.32		8627	4.34	5.73	3506	8.33	13.12
HE4	2000-3000000	pg/mL	55766	11.42	9.39	295050	4.28	4.45	55043	1.9		2288	6.47	7.67	2590	11.67	8.86
Prolactin	500-600000	pg/mL	11952	7.86	6.96	60823	3.43	4.13	10863	4.49		12191	3.31	2.83	10602	6.1	4.71
Leptin	500-600000	pg/mL	13001	12.79	7.06	64533	2.79	2.4	11020	5.73		14622	5.67	3.18	9145	6.58	3.4
OPN	2000-3000000	pg/mL	57576	9.61	8.58	240688	4.93	9.29	42845	3.77		75488	3.61	3.35	10700	5.59	4.85
HGF	100-120000	pg/mL	2546	9.92	8.68	13471	3.23	3.3	2171	2.51		894	2.82	2.53	436	7.6	8.8
MIF	100-120000	pg/mL	2263	10.84	10.06	12332	5.12	5.99	2220	5.19		876	2.06	1.86	517	11.4	17.25
sFas	100-150000	pg/mL	2533	7.15	6.72	13212	3.91	3.47	2775	3.94		4005	3.54	3.07	2472	4.88	4.34
sFasL	50-60000	pg/mL	1245	6.35	6.49	6023	2.9	3.15	1093	5.13		< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	253	8.52	8.64	1269	2.65	2.72	220	3.73		72	4.22	4.71	98	6.19	6.44
VEGF	50-60000	pg/mL	1146	16.04	11.12	6155	4.61	3.56	1100	2.88		< MR	< MR	< MR	<MR	< MR	< MR
IL-6	2-3000	pg/mL	54	11.72	12.01	268	6.35	6.11	54	1.79		21	6.25	7.44	< MR	< MR	< MR
IL-8	5-6000	pg/mL	124	7.8	9.02	668	6.56	9.49	111	3.94		26	5.05	9.13	6.70	6.22	9.43
TNFα	5-6000	pg/mL	117	10.43	10.35	583	3.68	3.8	111	4.86		16	5.27	9.14	7.85	5.41	4.61
TGFα	10-12000	pg/mL	238	8.86	8.94	1210	3.78	4.05	221	4.91		27	3.99	9.09	18	5.32	9.69
FGF2	50-50000	pg/mL	1104	20.18	14.17	5708	2.97	3.07	1068	2.04		68	9.63	13.35	80	7.72	7.97
SCF	20-30000	pg/mL	603	8.04	7.66	2951	4.39	4.32	549	3.96		79	6.49	8.93	59	8.5	15.12

Intra-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of all assays 1-10 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

Dilution linearity

The 50% dilutions of pool 1 samples were run in each of the five plates. The dilution of 20 biomarkers fell into the accepted recovery of 70%-130%. Range of all markers was between 53.19% (β-HCG) and 136.24% (FGF2) (Figure 1). Here, concentration levels calculated by extrapolation were included. VEGF was the only biomarker without any calculable levels of concentration.

Different storage conditions

Samples centrifuged and measured after being stored for 6 h at room temperature (25 ℃) yielded a median recovery of 95.0% [range: 84.5% (IL-6)-204.4% (MIF)], while a centrifugation after 24 h showed a stronger effect on some biomarkers with a median recovery of 108.2% ranging from 92.8% for TRAIL to 1453.3% for IL-8. However, only two biomarkers after 6 h and three biomarkers after 24 h failed the accepted range of recovery (IL-8 and MIF) (Figure 2).

Table 3 Inter-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2505	10.45	3.92	10933	8.88	7.62	2150	11.53	4.15	38897	4.53	6.58	466	15.14	14.43
AFP	500-600000	pg/mL	11980	13.92	5.32	54865	12.72	5.09	10703	12.14	3.43	455084	8.59	20.68	1598	34	66.04
Total PSA	50-60000	pg/mL	1230	15.82	5.11	5880	11.51	11.21	1057	8.61	0.32	7762	7.88	4.59	< MR	< MR	< MR
CA 15-3	0.5-600	U/mL	12	18.57	6.38	57	21.29	2.85	11	20.52	1.79	29	16.48	8.48	24	27.78	21.2
CA 19-9	2-3000	U/mL	61	20.3	4.79	292	12.19	4.58	54	11.44	2.19	33	17.49	7.29	10	19.24	23.19
CA 125	2-4000	U/mL	71	13.39	3.23	347	11.19	3.9	68	11.73	1.91	302	6.26	8.27	5.53	22.25	36.3
β-HCG	0.2-400	mU/mL	8	17.8	4.39	39	16.3	1.48	7	15.48	2.41	0.65	4.49	61.53	< MR	< MR	< MR
CYFRA 21-1	500-900000	pg/mL	29454	16.78	27.37	155151	15.64	40.96	16614	42.71	1.14	9724	38.02	50	3506	21.25	30.8
HE4	2000-3000000	pg/mL	55648	27.52	5.48	291745	23.45	3.46	55123	23.76	2.74	2276	14.67	63.85	< MR	< MR	< MR
Prolactin	500-600000	pg/mL	11868	26.35	4.16	60195	15.98	2.69	10922	14.69	1.97	12057	21.22	10.08	10765	35.99	20.91
Leptin	500-600000	pg/mL	13056	23.59	2.44	62955	14.97	3.92	10971	15.63	1.81	13430	18.98	8.79	8468	36.1	23.77
OPN	2000-3000000	pg/mL	53895	45.75	4.01	236975	29.04	4.62	43171	31.41	2.38	73954	46.72	8.5	10870	85.54	22.7
HGF	100-120000	pg/mL	2452	13.41	12.19	12766	16.96	8.28	2154	20.58	1.7	785	7.96	11.57	283	10.19	20.14
MIF	100-120000	pg/mL	2230	19.69	7.89	11061	14.32	4.89	2209	12.31	1.68	574	8.59	13.68	513	63.56	62.9
sFas	100-150000	pg/mL	2486	27.52	6.36	12705	21.86	6.83	2779	26.23	1.37	3548	30.07	7.78	2217	51.45	22.99
sFasL	50-60000	pg/mL	1219	16.69	4.68	5898	20.4	2.92	1091	17.72	1.62	< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	252	13.53	4.72	1233	13.51	4.09	221	15.17	1.85	65	10.05	9.26	92	27.2	23.98
VEGF	50-60000	pg/mL	1127	25.4	4.27	6110	24.51	8.87	1115	18.99	3.76	< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	49	14.49	9.34	226	22.21	17.12	53	18.81	4	16	12.3	14.64	< MR	< MR	< MR
IL-8	5-6000	pg/mL	122	8.85	5.21	605	11.32	3.73	110	9.31	1.98	33	9.31	8.37	6.71	25.35	40.54
TNFα	5-6000	pg/mL	114	21.44	5.52	562	16.31	6.81	110	16.78	2.28	13	19.54	8.14	6.41	26.96	40.75
TGFα	10-12000	pg/mL	237	16.15	6.26	1180	13.07	3.33	221	15.5	1.97	24	16.87	23.88	18	24.27	32.78
FGF2	50-50000	pg/mL	1101	11.17	8.98	5571	13.86	5.71	1066	12.75	3.89	55	11.36	68.81	79	19.8	40.04
SCF	20-30000	pg/mL	599	15.96	10.08	2813	13.38	4.75	550	14.69	1.93	58	11.5	10.22	50	24.27	22.5

Inter-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of assays 1-5 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

When samples were directly centrifuged after venous puncture and subsequently stored at 4 ℃ up to 48 h before measurements, biomarker levels showed relatively stable results. When storing the samples at room temperature for 6, 24 and 48 h before testing, stronger alterations were observed. Indeed, two, 0 and 16 biomarkers, respectively, failed the 70%-130% range of recovery. Detailed results are shown in Figures 3 and 4.

Testing in EDTA-plasma instead of serum samples did generally not affect the levels of biomarkers resulting in a median recovery of 110.8% [range: 60.7% (HGF)-272% (OPN)]. Here, four biomarkers failed the range of recovery (Figure 5).

DISCUSSION

Launching the multiplex magnetic bead assay into clinical routine and thus providing an insight of diverse corporal processes by means of biomarkers would greatly support

Table 4 Extended inter-assay imprecision

Biomarker	Measuring range	Unit	QC 1			QC 2			St 5			Pool 1			Pool 2		
			Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%	Conc Mean	FI Mean CV%	Conc Mean CV%
CEA	100-120000	pg/mL	2545	13.96	28.08	12727	20.57	25.67	2146	13.34	4.59	39391	6.94	7.38	560	14.32	25.77
AFP	500-600000	pg/mL	12038	23.54	20.99	56960	19.11	10.29	10754	21.43	3.25	493993	12.38	18.42	1489	33.13	63.64
Total PSA	50-60000	pg/mL	1421	34.29	31.87	6172	17.44	13.78	1052	18.72	3.67	8246	16.03	12.46	68	130.09	151.66
CA 15-3	0.5-600	U/mL	12	30.11	18.58	62	29.25	12.98	11	34.01	2.93	33	23.72	17.26	26	30.49	27.59
CA 19-9	2-3000	U/mL	64	22.71	17.91	329	11.98	19.1	54	19.2	2.71	36	15.18	26.88	10	16.18	36.06
CA 125	2-4000	U/mL	74	14.42	22.04	370	12.5	11.07	68	17.29	2.42	317	7.72	12.62	6.13	18.13	37.03
β-HCG	0.2-400	mU/mL	7.85	20.62	20.74	42	16.95	16.23	7	26.88	2.69	1.2	29.09	52.2	0.55	17.3	74.36
CYFRA 21-1	500-900000	pg/mL	24985	69.07	36.07	152731	81.41	52.97	16601	53.61	2.5	8627	50.45	55.77	3506	31.82	30.8
HE4	2000-3000000	pg/mL	55766	22.11	21.46	295050	19.92	10.63	55043	22.45	2.1	2288	19.7	68.01	2590	22.73	54.77
Prolactin	500-600000	pg/mL	11952	23.76	21.49	60823	13.52	9.33	10863	14.81	2.05	12191	17.16	20.41	10602	29.43	28.46
Leptin	500-600000	pg/mL	13001	29.19	15.47	64533	19.61	8.82	11020	23.3	1.88	14622	16.75	18.61	9145	31.06	27.03
OPN	2000-3000000	pg/mL	57576	58.11	28.1	240688	24.08	13.88	42845	25.18	5.71	75488	41.77	25.01	10700	62.83	47.91
HGF	100-120000	pg/mL	2546	15.19	20.7	13471	19.05	12.85	2171	22.65	2.04	894	7.18	27.83	436	29.6	49.81
MIF	100-120000	pg/mL	2263	46.52	45.1	12332	20.48	18.05	2220	23.01	2.93	876	62.88	48.48	517	45.1	47.35
sFas	100-150000	pg/mL	2533	27.15	18.62	13212	20.06	12.63	2775	27.38	1.77	4005	29.39	21.39	2472	39.44	28.26
sFasL	50-60000	pg/mL	1245	19.09	20.91	6023	21.37	6.63	1093	22.37	1.98	< MR	< MR	< MR	< MR	< MR	< MR
TRAIL	10-12000	pg/mL	253	20.17	21.78	1269	17.13	10.13	220	21.55	2.05	72	11.04	32.6	98	26.7	35.57
VEGF	50-60000	pg/mL	1146	46.21	23	6155	45.49	8.97	1100	43.93	3.26	< MR	< MR	< MR	< MR	< MR	< MR
IL-6	2-3000	pg/mL	54	17.46	26.94	268	17.39	23.92	54	24.51	5.54	21	14.19	41.05	< MR	< MR	< MR
IL-8	5-6000	pg/mL	124	75.72	21.22	668	45.79	22.2	111	30.7	3.13	26	36.91	38.62	6.70	90.89	40.54
TNFα	5-6000	pg/mL	117	18.6	23.55	583	16.6	11.16	111	19.41	2.52	16	15.5	28.1	7.85	22.35	49.24
TGFα	10-12000	pg/mL	238	18.16	21.43	1210	19.49	7.74	221	22.29	2.56	27	17.64	45.12	18	19.22	48.85
FGF2	50-50000	pg/mL	1104	25.12	23.52	5708	17.67	9.9	1068	19.05	3.55	68	9.43	49.91	80	15.21	32.41
SCF	20-30000	pg/mL	603	12.44	19.52	2951	16.66	11.26	549	17.88	2.01	79	31.9	40.75	59	22.35	32.17

Inter-assay imprecision results for all 24 tested markers based on FI as well as observed concentration (Obs Conc) results of all assays 1-10 for synthetic quality controls QC 1, QC 2, standard 5 as well as for physiological serum pools 1 and 2. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

tumor diagnostics and differential diagnosis[20,21]. Here, an appropriate pre-analytical evaluation is indispensible in order to obtain reliable clinical results in the future[2,3].

As well-known for many research-use-only (RUO)-assays, there often is only limited data available concerning the methodological performance despite being distributed and used for study and research purposes[20-22]. This is quite relevant for the scientific community as the same names of the parameters are used in these compound tests as in regular *in-vitro* diagnostic (IVD) labeled assays which are commonly used in clinical laboratory routine diagnostics for which the methodical quality and clinical validity has been investigated thoroughly in most cases.

Certainly, the assay of the present study has to be perceived as a complex method consisting of as many different tests as markers are included. It seems to be quite challenging for manufacturers to optimize all markers in a multiparametric platform and to avoid interactions between them. This is all the more obvious by the conspicuous variety of the measured biomarkers regarding their chemical and physical characteristics. Thus, to evaluate the measurement quality of this assay the single markers have to be interpreted independently.

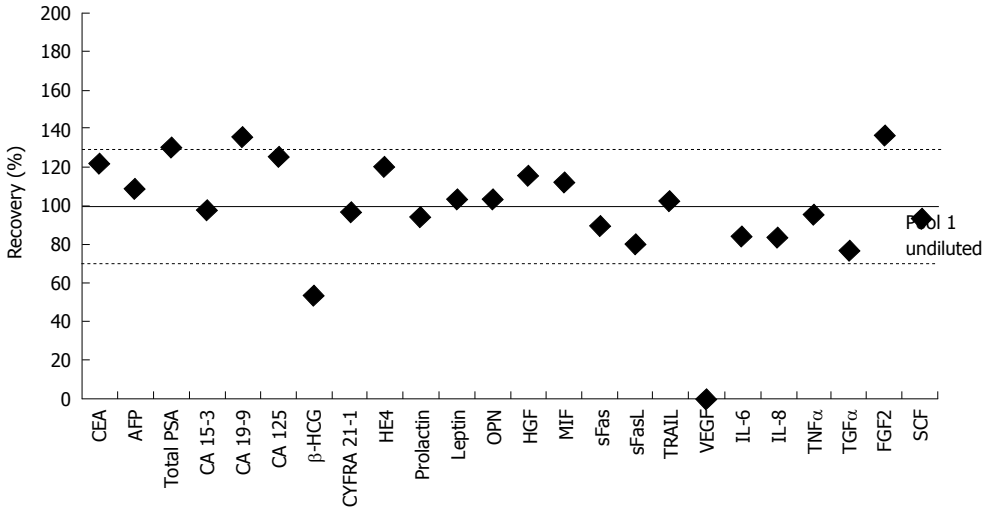

Figure 1 Dilution recovery. Recoveries of a 50% dilution of serum pool 1 for all 24 biomarkers. The values are observed concentration-based and the corresponding measured undiluted pool 1 values were taken as reference. Horizontal lines depict the acceptable range of recovery. The dilution of VEGF fell below the measuring range. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

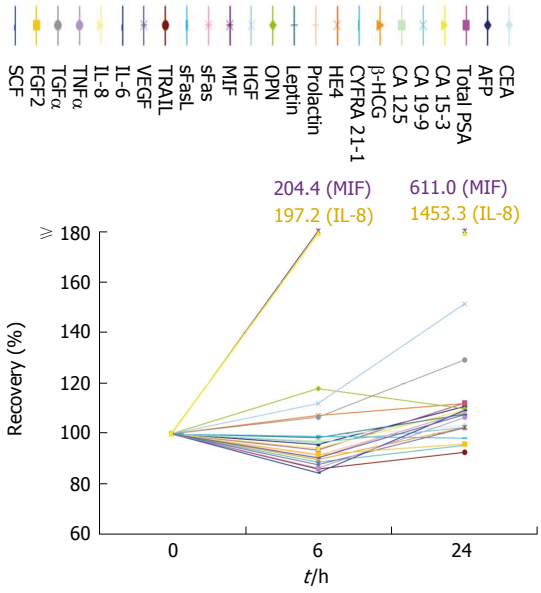

Figure 2 Preanalytical factors: Storage before centrifugation. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored at room temperature for 0, 6 and 24 h before centrifugation, definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

All in all, ten plates were run. The first five kits were ordered in one batch and applied subsequently under strictly identical conditions. The following plates were ordered and run six months later under the same standards of procedure. However, we perceived a striking discrepancy when comparing the results of the first five and the last five plates. As the lot number was the same, we assume an influence of surrounding conditions, *e.g.*, room temperature, pipettes used and different laboratory staff constellation. In order to represent accurate and relevant results in our main method evaluation, we divided our evaluation into two steps: First, we considered only the first five assays as they were done under homogeneous conditions. In the second step, an overall analysis of all assays was performed. Nevertheless, this critical fact elucidates once more the importance of standardized procedures in clinical routine laboratories, the application of internal quality controls and the participation in external quality assessment programs[3,18].

In general, the assay showed an acceptable intra- and inter-assay imprecision. Apart from the synthetic internal controls QC 1 and QC 2 we included the physiological external control samples pool 1 and pool 2 as a further reference source. In comparison to the internal controls QC 1 and QC 2 this resulted in an interesting finding. As expected, the method precision was slightly more accurate for the synthetic samples. Although evidently the composition of serum pools is more complex, this physiological type of quality control is more relevant as it reflects the situation of the clinical samples more accurately. Nevertheless, the comparability between synthetic and physiological samples was still given. However, we perceived higher variation in samples with lower con-

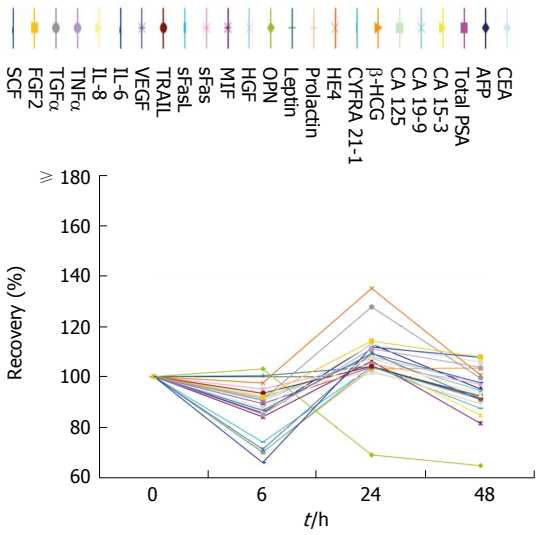

Figure 3 Preanalytical factors: Storage after centrifugation at 4 ℃. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored for 0, 6, 24 and 48 h after centrifugation at 4 ℃ before definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

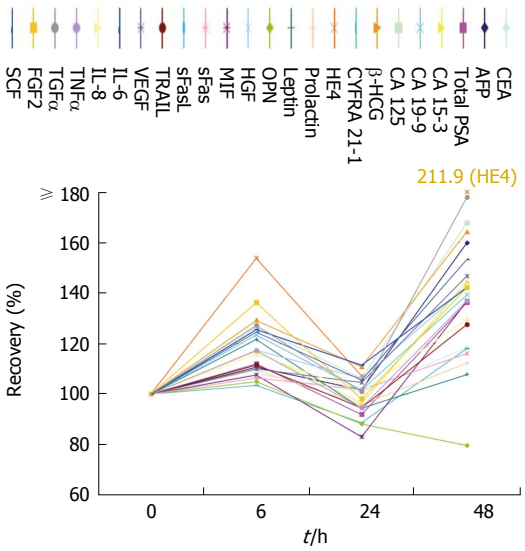

Figure 4 Preanalytical factors: Storage after centrifugation at room temperature. Behavior of biomarkers undergoing different pre-analytical conditions: Blood samples were stored for 0, 6, 24 and 48 h after centrifugation at room temperature before definitive storage at -80 ℃ and measurement. Values are FI-based and depicted as recoveries corresponding to the values of directly centrifuged and frozen serum as reference. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

centrations of the biomarkers, particularly seen in the imprecision results of pool 2 where most marker concentrations were below or at the lower end of the accepted range.

Comparing the precision data provided by the manufacturer (range: 4.9% to 15.0% and 4.1% to 16.2% for intra- and inter-assay imprecision, respectively) with our results a very good accordance concerning the intra-assay results was observed.

The inter-assay imprecision is partly higher in our tests. However, for most markers we could achieve the corresponding coefficients of variation considering the results of the quality controls provided by the manufacturer. CYFRA 21-1, a highly valuable tumor marker for non small cell lung cancer[16,23,24], was found to be the only marker exceeding the 20% limit of CV concurring with the given imprecision by the manufacturer where it also yields the maximum coefficient of variation in the panel. As the marker concentration is within the appropriate range and the variation is found to be not acceptable we assume a non-applicability of this biomarker in the assessed method. In our higher concentrated pool 1, five markers were found to be measured with a non-acceptable CV. Close observation of these biomarkers revealed that the observed concentrations were very low, even below the first standard (such as for FGF2) indicating only limited clinical relevance in these cases. In conclusion, with an inter-assay imprecision in a reasonable range between 4.59% (total PSA)-23.88% (TGFα), this is a rather satisfying result.

Comparing the values of variance of fluorescence intensity and observed concentration revealed some striking discrepancies. This phenomenon is obviously based on the transformation of measured fluorescence intensity into biomarker concentration, which is neither linear nor predetermined to be equal in all the tests, but corresponding to the course of the standard curve calculated anew for every single test. FI values are matched to the concentrations that are provided by the standard curve. Thus, the same FI values in two different plates could lead to two different concentrations and are therefore less comparable when it comes to a crossover comparison between the plates. This phenomenon is shown in the attached Table 3 comparing FI values and observed concentration-based CVs. Hence, the inter-assay imprecision based on concentration values provided a more relevant result for the methodological evaluation.

Testing the dilution linearity, our study yields satisfying results with a tendency to a recovery in the upper field of the defined acceptable range.

Within the Bio-Plex® 200 System, it is possible to vary the minimal number of events needed for measurement. With 50 events as minimum per bead we chose a

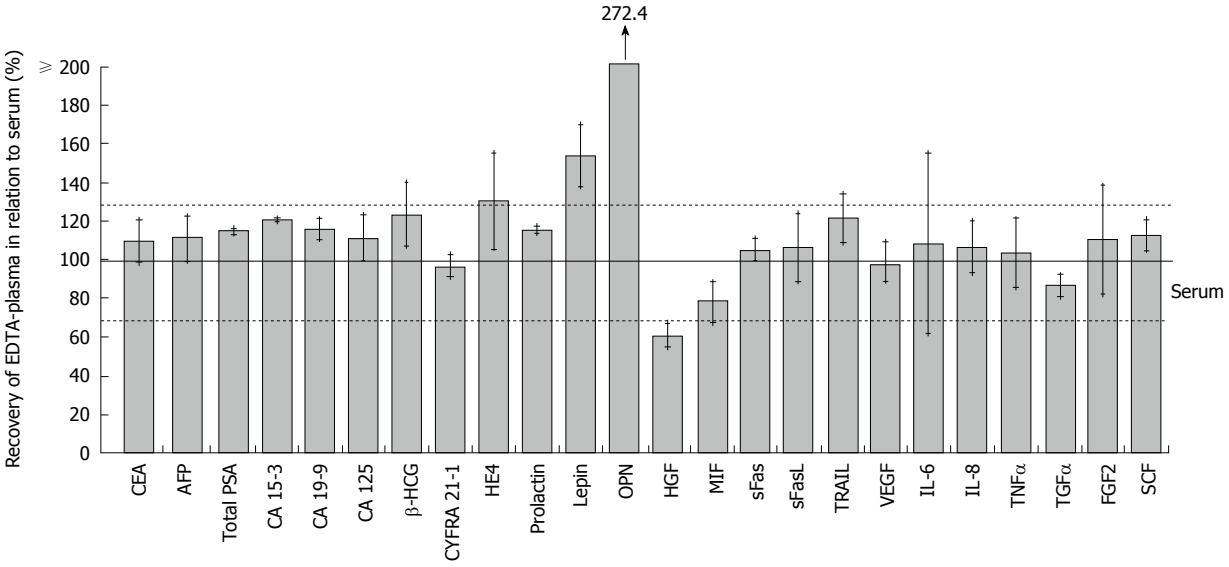

Figure 5 Comparison between serum and EDTA-plasma. Measured serum levels are defined as 100% recovery. The acceptable range is indicated by the horizontal lines and standard deviation of the recoveries is given for each marker. AFP: Alpha-fetoprotein; CA 125: Cancer antigen 125; CEA: Carcinoembryonic antigen; CV: Coefficient of variation; CYFRA 21-1: Cytokeratine 19-fragment; FGF2: Fibroblast growth factor-2; FI: Fluorescent intensity; HE4: Human epididymis protein 4; HGF: Hepatocyte growth factor; IL-6: Interleukin-6; MIF: Macrophage migration inhibitory factor; MR: Measuring range; Conc: Concentration; OPN: Osteopontin; QC 1: Quality control 1; SCF: Stem cell factor; sFas: Soluble Fas; sFasL: Soluble Fas-ligand; TGFα: Transforming growth factor-α; TNFα: Tumor necrosis factor-α; total PSA: Total prostate-specific antigen; TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; VEGF: Vascular endothelial growth factor; β-HCG: β-human chorionic gonadotropin.

compromise between a time-effective measurements and sufficiently precise results. Obviously, the accuracy of measurements increases with higher number of events. Nevertheless, our findings could achieve acceptable CV values in most cases despite the low minimum number of events set. Therefore, we did not compare absolute number of detected events and their calculated CVs.

The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 offers a wide spectrum of applicable biomarkers. However, it is obviously neither clinically relevant nor cost-effective to apply the complete panel in diagnostics. After definition of the relevant markers for each tumor type, the panel must be focused and applied as a biomarker pattern of clinical interest depending on the contemplated entity of disease.

For implementation into clinical diagnostics, further studies evaluating its performance in a large cohort of cancer patients and appropriate control groups, which are relevant for differential diagnosis, *i.e.*, healthy individuals and patients with organ related benign disease, are definitely required. Currently, we are performing such clinical validation studies with cohorts of patients suffering from gastrointestinal, gynecological and urological cancers.

Apart from the method quality itself, preanalytical handling of samples prior to analysis in the laboratory can influence the final results, as is known for several research and routine parameters[25-27]. Hence, we examined the stability of the tested markers. As often observed in the clinical routine, samples are not directly transferred to the central laboratory and instead remain exposed to room temperature without centrifugation. In order to depict this highly relevant situation, samples were centrifuged after 6 and 24 h. However, most markers remained stable, except MIF and IL-8. These two markers presented a considerable increase in marker levels. Our findings for IL-8 agree with the recommendations made by Hoch *et al*[28] to centrifuge the blood samples within less than 2 h to avoid interactions between IL-8 and blood cells. An increase of MIF levels in samples, which were not directly prepared, is also predescribed by Sobierajski *et al*[29]. These pre-analytical facts must be observed by the clinicians as prolonged storage before processing could lead to fatal misinterpretation in these markers.

Sample storage up to 48 h after centrifugation at a temperature of 4 ℃ showed a good outcome for all markers except OPN, which presented a decline after 6 h storage.

Storage at 25 ℃ after centrifugation showed a stronger effect on marker levels. While stability is given until 24 h, a more or less increase of marker concentrations can be observed after longer storage time. For example, the recovery of HE4 rises over 200% after 48 h. Again, OPN is the only marker showing a decline to nearly 80% in recovery supporting our above mentioned findings. Also, Cristaudo *et al*[30] found an instable performance of OPN after storage of serum samples at room temperature.

Furthermore, we observed good comparability between serum and EDTA-plasma samples. Only OPN and leptin-recoveries exceeded the 130% mark of the accepted range. These results correspond with the findings of Lanteri *et al*[31] and Gröschl *et al*[32] where an increase of these biomarkers in plasma samples compared with serum samples was observed. HGF presented a recovery of 60.7% as the unique marker undergoing the accepted 70% mark, also concurring with previous HGF stability analysis[33].

In our study, the evaluated method is demonstrated

to be a stable and precise tool for detection of most biomarkers included in the kit. Unfortunately, for CYFRA 21-1, the method did not achieve acceptable inter-assay precision values. This should be further investigated. In general, we recommend that "research use only"-tests are assessed before implementation into further research and clinical routine. Here, certain preconditions, such as ordering tests in a batch, use of physiological quality controls in addition to the provided control samples as well as relevant pre-analytical aspects of some markers, should be observed.

All in all, this study shows that the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1 could offer new diagnostic perspectives while further studies are necessary to show its clinical applicability, usefulness and comparability with established routine assays.

ACKNOWLEDGMENTS

We are grateful for access to the Biorad and Luminex instruments of the laboratory of Professor Knolle and the laboratory of Professor Hornung at the University Hospital Bonn, where the analyses were performed. This work is part of the doctoral theses of Hermann N and Dreßen K.

COMMENTS

Background

Cancer is a global health problem resulting in about eight million deaths each year. Tumor markers as additional diagnostic tools, which are easily assessable in blood, have been launched in the last century and with them a large variety of detecting methods. Of these, enzyme-linked immunosorbent assays are the standard methods in clinical routine today, while multiplexing has become interesting for cancer research as it is a quick, cheap, less-volume-wasting, easy-to-handle but still precise tool for parallel measurement of multiple markers. This goes hand in hand with the fact that nowadays cancer is perceived as a complex disease involving multiple processes. The authors investigated the MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel 1, purchased from Millipore, which was specially designed for cancer diagnostics, on its methodical performance.

Research frontiers

Most striking is the fact that many different assays are used for research purpose, although they are not that strictly proven as established methods for *in-vitro* diagnostics. However, research results are assigned to a clinical setting. Therefore, a thorough methodical investigation using physiological samples has to be performed.

Innovations and breakthroughs

Previous investigations of the multiplex technology used by Millipore showed good correlations to single ELISAs of each tested biomarker. However, here only a few markers were tested in parallel. Furthermore, this specially designed kit with the possibility to measure 24 biomarkers from the areas of angiogenesis, immunology, apoptosis as well as established and auspicious tumor markers has not been tested on its entirety regarding methodological performance and stability. In the investigations, the authors used serum pools as physiological control samples and most markers showed good results.

Applications

This study allows other users to assess the quality and applicability of the assay and to conduct further clinical studies on methodically solid bedrock.

Terminology

All substances that can be measured in cancer patients and which reveal a malignant disease or contribute to its prognosis or treatment are called tumor markers. Multiplexing is one of the detection methods for tumor markers which are assessed in body fluids and it allows the parallel measurement of multiple markers. An ELISA is an enzyme-linked immunosorbent assay which is another detection method for tumor markers but limited to the measurement of only one marker per test. Both are antibody-based detection procedures and enzymatic color-reactions are used to quantify the results.

Peer review

The manuscript is very well presented and highlights factors influencing the measurements of the key performance indicators of the multiplex cancer biomarker panel, which constitutes a highly interesting study. The findings from the comparison of the critical measurement parameters between physiological sera in parallel with synthetic internal controls will be of particular interest to a wide audience. The technical details are clearly defined and the interpretations are thorough with robust scientific conclusions.

REFERENCES

1 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29 [PMID: 22237781 DOI: 10.3322/caac.20138]
2 **Bretthauer M**, Kalager M. Principles, effectiveness and caveats in screening for cancer. *Br J Surg* 2013; **100**: 55-65 [PMID: 23212620 DOI: 10.1002/bjs.8995]
3 **Sturgeon CM**, Hoffman BR, Chan DW, Ch'ng SL, Hammond E, Hayes DF, Liotta LA, Petricoin EF, Schmitt M, Semmes OJ, Söletormos G, van der Merwe E, Diamandis EP. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for use of tumor markers in clinical practice: quality requirements. *Clin Chem* 2008; **54**: e1-e10 [PMID: 18606634 DOI: 10.1373/clinchem.2007.094144]
4 **Sturgeon CM**, Duffy MJ, Hofmann BR, Lamerz R, Fritsche HA, Gaarenstroom K, Bonfrer J, Ecke TH, Grossman HB, Hayes P, Hoffmann RT, Lerner SP, Löhe F, Louhimo J, Sawczuk I, Taketa K, Diamandis EP. National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines for use of tumor markers in liver, bladder, cervical, and gastric cancers. *Clin Chem* 2010; **56**: e1-48 [PMID: 20207771 DOI: 10.1373/clinchem.2009.133124]
5 **Locker GY**, Hamilton S, Harris J, Jessup JM, Kemeny N, Macdonald JS, Somerfield MR, Hayes DF, Bast RC. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 2006; **24**: 5313-5327 [PMID: 17060676 DOI: 10.1200/JCO.2006.08.2644]
6 **Holdenrieder S**, Stieber P. Circulating apoptotic markers in the management of non-small cell lung cancer. *Cancer Biomark* 2010; **6**: 197-210 [PMID: 20660965]
7 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
8 **Kellar KL**, Douglass JP. Multiplexed microsphere-based flow cytometric immunoassays for human cytokines. *J Immunol Methods* 2003; **279**: 277-285 [PMID: 12969567 DOI: 10.1016/S0022-1759(03)00248-5]
9 **Chowdhury F**, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. *J Immunol Methods* 2009; **340**: 55-64 [PMID: 18983846 DOI: 10.1016/j.jim.2008.10.002]
10 **Kim YW**, Bae SM, Lim H, Kim YJ, Ahn WS. Development of multiplexed bead-based immunoassays for the detection of early stage ovarian cancer using a combination of serum biomarkers. *PLoS One* 2012; **7**: e44960 [PMID: 22970327 DOI: 10.1371/journal.pone.0044960]
11 **Nolen BM**, Lokshin AE. Biomarker testing for ovarian cancer: clinical utility of multiplex assays. *Mol Diagn Ther* 2013; **17**: 139-146 [PMID: 23552992 DOI: 10.1007/s40291-013-0027-6]
12 **Park HD**, Kang ES, Kim JW, Lee KT, Lee KH, Park YS, Park JO, Lee J, Heo JS, Choi SH, Choi DW, Kim S, Lee JK, Lee SY. Serum CA19-9, cathepsin D, and matrix metalloproteinase-7

as a diagnostic panel for pancreatic ductal adenocarcinoma. *Proteomics* 2012; **12**: 3590-3597 [PMID: 23065739 DOI: 10.1002/pmic.201200101]
13 **Krishhan VV**, Khan IH, Luciw PA. Multiplexed microbead immunoassays by flow cytometry for molecular profiling: Basic concepts and proteomics applications. *Crit Rev Biotechnol* 2009; **29**: 29-43 [PMID: 19514901 DOI: 10.1080/07388550802688847]
14 **Ellington AA**, Kullo IJ, Bailey KR, Klee GG. Antibody-based protein multiplex platforms: technical and operational challenges. *Clin Chem* 2010; **56**: 186-193 [PMID: 19959625 DOI: 10.1373/clinchem.2009.127514]
15 **Horvath AR**, Kis E, Dobos E. Guidelines for the use of biomarkers: principles, processes and practical considerations. *Scand J Clin Lab Invest Suppl* 2010; **242**: 109-116 [PMID: 20515288 DOI: 10.3109/00365513.2010.493424]
16 **Molina R**, Holdenrieder S, Auge JM, Schalhorn A, Hatz R, Stieber P. Diagnostic relevance of circulating biomarkers in patients with lung cancer. *Cancer Biomark* 2010; **6**: 163-178 [PMID: 20660962 DOI: 10.3233/CBM-2009-0127]
17 **Moore RG**, Jabre-Raughley M, Brown AK, Robison KM, Miller MC, Allard WJ, Kurman RJ, Bast RC, Skates SJ. Comparison of a novel multiple marker assay vs the Risk of Malignancy Index for the prediction of epithelial ovarian cancer in patients with a pelvic mass. *Am J Obstet Gynecol* 2010; **203**: 228.e1-228.e6 [PMID: 20471625 DOI: 10.1016/j.ajog.2010.03.043]
18 **Dancey JE**, Dobbin KK, Groshen S, Jessup JM, Hruszkewycz AH, Koehler M, Parchment R, Ratain MJ, Shankar LK, Stadler WM, True LD, Gravell A, Grever MR. Guidelines for the development and incorporation of biomarker studies in early clinical trials of novel agents. *Clin Cancer Res* 2010; **16**: 1745-1755 [PMID: 20215558 DOI: 10.1158/1078-0432.CCR-09-2167]
19 **Sackett DL**, Haynes RB. The architecture of diagnostic research. *BMJ* 2002; **324**: 539-541 [PMID: 11872558]
20 **Fu Q**, Zhu J, Van Eyk JE. Comparison of multiplex immunoassay platforms. *Clin Chem* 2010; **56**: 314-318 [PMID: 20022982 DOI: 10.1373/clinchem.2009.135087]
21 **Bastarache JA**, Koyama T, Wickersham NE, Mitchell DB, Mernaugh RL, Ware LB. Accuracy and reproducibility of a multiplex immunoassay platform: a validation study. *J Immunol Methods* 2011; **367**: 33-39 [PMID: 21277854 DOI: 10.1016/j.jim.2011.01.005]
22 **Köhler K**, Seitz H. Validation processes of protein biomarkers in serum--a cross platform comparison. *Sensors* (Basel) 2012; **12**: 12710-12728 [PMID: 23112739 DOI: 10.3390/s120912710]
23 **Barak V**, Goike H, Panaretakis KW, Einarsson R. Clinical utility of cytokeratins as tumor markers. *Clin Biochem* 2004; **37**: 529-540 [PMID: 15234234 DOI: 10.1016/j.clinbiochem.2004.05.009]
24 **Holdenrieder S**, von Pawel J, Dankelmann E, Duell T, Faderl B, Markus A, Siakavara M, Wagner H, Feldmann K, Hoffmann H, Raith H, Nagel D, Stieber P. Nucleosomes and CYFRA 21-1 indicate tumor response after one cycle of chemotherapy in recurrent non-small cell lung cancer. *Lung Cancer* 2009; **63**: 128-135 [PMID: 18571761 DOI: 10.1016/j.lungcan.2008.05.001]
25 **Yoshimura T**, Fujita K, Kawakami S, Takeda K, Chan S, Beligere G, Dowell B. Stability of pro-gastrin-releasing peptide in serum versus plasma. *Tumour Biol* 2008; **29**: 224-230 [PMID: 18781094 DOI: 10.1159/000152940]
26 **Lehner J**, Wittwer C, Fersching D, Siegele B, Holdenrieder S, Stoetzer OJ. Methodological and preanalytical evaluation of an HMGB1 immunoassay. *Anticancer Res* 2012; **32**: 2059-2062 [PMID: 22593488]
27 **Holdenrieder S**, Mueller S, Stieber P. Stability of nucleosomal DNA fragments in serum. *Clin Chem* 2005; **51**: 1026-1029 [PMID: 15914786 DOI: 10.1373/clinchem.2005.048454]
28 **Hoch RC**, Schraufstätter IU, Cochrane CG. In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptors. *J Lab Clin Med* 1996; **128**: 134-145 [PMID: 8765209 DOI: 10.1016/S0022-2143(96)90005-0]
29 **Sobierajski J**, Hendgen-Cotta UB, Luedike P, Stock P, Rammos C, Meyer C, Kraemer S, Stoppe C, Bernhagen J, Kelm M, Rassaf T. Assessment of macrophage migration inhibitory factor in humans: protocol for accurate and reproducible levels. *Free Radic Biol Med* 2013; **63**: 236-242 [PMID: 23707455 DOI: 10.1016/j.freeradbiomed.2013.05.018]
30 **Cristaudo A**, Foddis R, Bonotti A, Simonini S, Vivaldi A, Guglielmi G, Ambrosino N, Canessa PA, Chella A, Lucchi M, Mussi A, Mutti L. Comparison between plasma and serum osteopontin levels: usefulness in diagnosis of epithelial malignant pleural mesothelioma. *Int J Biol Markers* 2010; **25**: 164-170 [PMID: 20878622]
31 **Lanteri P**, Lombardi G, Colombini A, Grasso D, Banfi G. Stability of osteopontin in plasma and serum. *Clin Chem Lab Med* 2012; **50**: 1979-1984 [PMID: 22718644 DOI: 10.1515/cclm-2012-0177]
32 **Gröschl M**, Wagner R, Dörr HG, Blum W, Rascher W, Dötsch J. Variability of leptin values measured from different sample matrices. *Horm Res* 2000; **54**: 26-31 [PMID: 11182632 DOI: 10.1159/000053226]
33 **Nayeri F**, Brudin L, Nilsson I, Forsberg P. Sample handling and stability of hepatocyte growth factor in blood samples. *Cytokine* 2002; **19**: 201-205 [PMID: 12297114 DOI: 10.1006/cyto.2002.1050]

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