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EXPERT OPINION

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Recent patents and technology transfer for molecular diagnosis of β -thalassemia and other hemoglobinopathies

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Introduction: Biological tests and genetic analyses for diagnosis and characterization of hematological diseases in health laboratories are designed with the aim of meeting the major medical needs of hospitals and pharmaceutical companies involved in this field of applied biomedicine. Genetic testing approaches to perform diagnosis consist of molecular techniques, which should be absolutely reproducible, fast, sensitive, cheap, and portable.

Areas covered: Biological tests analyzed involve adult/newborn subjects, whereas genetic analyses involve adult thalassemia patients, newborns, embryos/fetuses (including non-invasive prenatal diagnosis), pre-implantation embryos, and pre-fertilization oocytes.

Expert opinion: The most recent findings in the diagnostic approach for β -thalassemias are related to three major fields of investigation: moving towards ultrasensitive methodologies for effective detection of the primary causative mutation of β -thalassemia, including the development of polymerase chain reaction-free approaches and non-invasive prenatal diagnosis; comparing analyses of the genotype of β -thalassemia patients to high-HbF-associated polymorphisms; introducing whole genome association assays and next-generation sequencing. All these issues should be considered and discussed in the context of several aspects, including regulatory, ethical and social issues. DNA sequence data aligned with the identification of genes central to the induction, development, progression, and outcome of β -thalassemia will be a key point for directing personalized therapy.

Keywords: molecular diagnosis, technology transfer, thalassemia

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1. Introduction

β -Thalassemias are a group of hereditary hematological diseases caused by >200 mutations of the human β -globin gene, leading to low or absent production of adult β -globin and excess of α -globin content in erythroid cells, and causing ineffective erythropoiesis and low or absent production of adult hemoglobin (HbA) [1]. Together with sickle cell anemia (SCA), thalassemia syndromes are the most important pathologies in developing countries, in which the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of a very high frequency of these genetic diseases in the population. This significantly contributes to drive changes in the distribution of carriers and affected people in relation to the migration of populations from endemic areas to countries where their prevalence in indigenous populations had been extremely low (USA, Canada, Australia, South America, the United Kingdom, France, Germany, Belgium, the Netherlands and,



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Article highlights.

- Molecular diagnosis of β -thalassemia has an important impact on the health system, on the management of patients, on personalized therapy.
- Patents on molecular methodologies are available for prenatal, prenatal, pre-implantation and pre-fertilization diagnosis of β -thalassemia.
- Non-invasive prenatal diagnosis is becoming a key issue in the context of molecular diagnosis of β -thalassemia.
- Molecular diagnosis of β -thalassemia should also include 'genetic modifiers' and associated polymorphism(s) enabling us to anticipate the severity of the disease.
- Personalized treatment of β -thalassemia patients will be in the next future based on molecular diagnosis, whole genome association and next-generation sequencing.

This box summarizes key points contained in the article.

more recently, Scandinavia) [2]. These deep changes have encouraged most of the health systems of these countries in facilitating access to the prevention and treatment services available for these HbA disorders. On the other hand, considering some limitations of the currently available diagnostic approaches and the urgent need for non-invasive methodologies for guiding personalized treatments, novel alternative options for molecular diagnosis are expected to reach the market shortly [3].

As far as relevant numbers concerning these pathologies, 80 – 90,000,000 carriers (thalassemias and SCA) are present worldwide and 300,000 – 500,000 children are born each year with severe homozygous states of these diseases (100,000 die early in the postnatal life). In Italy 2,500,000 carriers are present, about 4,500 patients affected by thalassemia major, about 1,500 by thalassemia intermedia and 600–700 by SCA [4].

Biological tests and genetic analyses for diagnosis and characterization of hematological diseases in health laboratories should be designed and developed with the aim of meeting the major medical needs of hospitals and pharmaceutical companies involved in this field of applied biomedicine. Basically, biological tests involve adult/newborn subjects, whereas genetic analyses, as summarized in **Figure 1**, involve adult thalassemia patients (**Figure 1A**) [5], newborns (**Figure 1A**) [6], embryos/fetuses (**Figure 1B**) [7–11], pre-implantation embryos (**Figure 1C**) [12,13] and pre-fertilization oocytes (**Figure 1D**) [14,15].

Genetic testing approaches to perform diagnosis consist of molecular techniques, that should be absolutely reproducible, fast, sensitive, cheap, and portable. For example, polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) are the two common techniques used on a single or two cells obtained via embryo biopsy during pre-implantation diagnosis [16]. They are useful mainly for diagnosis and/or prognosis, even though recent studies point out the need of genetic information for the development of personalized therapy [see the scheme reported in **Figure 2**].

2. Patents and patent applications in diagnosis of thalassemia: general considerations

The interest on efficient and sensitive diagnosis of thalassemia is associated with key intervention in the management of the thalassemia patients, especially in the fields of genetic counseling, non-invasive prenatal diagnosis, prognosis and personalized treatments. By searching for 'thalassemia diagnosis' >700 entries can be retrieved and analyzed. From this analysis, the following considerations should be made: i) molecular diagnosis of thalassemia is object of patenting using biological samples from different sources, such as peripheral blood, amniotic cells, fetal cells, single cells from pre-implanted embryos, and oocyte-associated polar bodies (**Figure 1**); ii) several patents concern the development of novel diagnostic methods; iii) information about companies interested in the technological transfer of these patents are available by looking at the section 'applicants'. It should be pointed out that in consulting patent Databases, a same patent might be identified in different entries (e.g., patent US8097405, published as a granted patent in 17 Jan 2012, has been published also as CA1223831, DE3382626, DE3382782, DE3382822, EP0097373, EP0285057, EP0285058, EP0285950, EP0286898, EP0302175, EP0618228 and US6992180) [17]; in this case, with only few exceptions, we gave arbitrarily priority to codes of issued US patents and US patent applications and WIPO (World Intellectual Property Organization), avoiding duplications. A further consideration is related to scientific validation and scientific merit. In this respect, within all the considered patent applications on diagnosis of thalassemia, only those based on suitable scientific publications in peer-reviewed journals have been included in the present review. A final introductory comment is related to the completeness of the list of patents and patent applications here reviewed. Despite the fact that the author put a great effort in presenting a balanced picture of the available entries, the possibility of absence of key patents cannot be excluded; in this case the author would like to present his deep apologize to the involved inventors and assignees.

A first example of molecular approach for the screening is the quantification of the α -globin and β -globin mRNAs. This strategy is based on the observation, obtained when the diagnosis of thalassemias was in its infancy, that β -thalassemias are characterized by an excess of α -globin chain expression, which can be detected by chromatography of globin chains. Huang *et al.* [18] developed a technique to diagnose the α - and β -thalassemia syndromes using the PCR to amplify cDNA copies of circulating erythroid mRNA. Quantitation of α -, β - and γ -globin mRNAs was performed by scintillation counting of ^{32}P -dCTP incorporated into specific globin cDNA bands and showed ratios of α/β -globin mRNA greater than 10-fold and greater than 5-fold increased in patients with β^0 and β^+ -thalassemia,

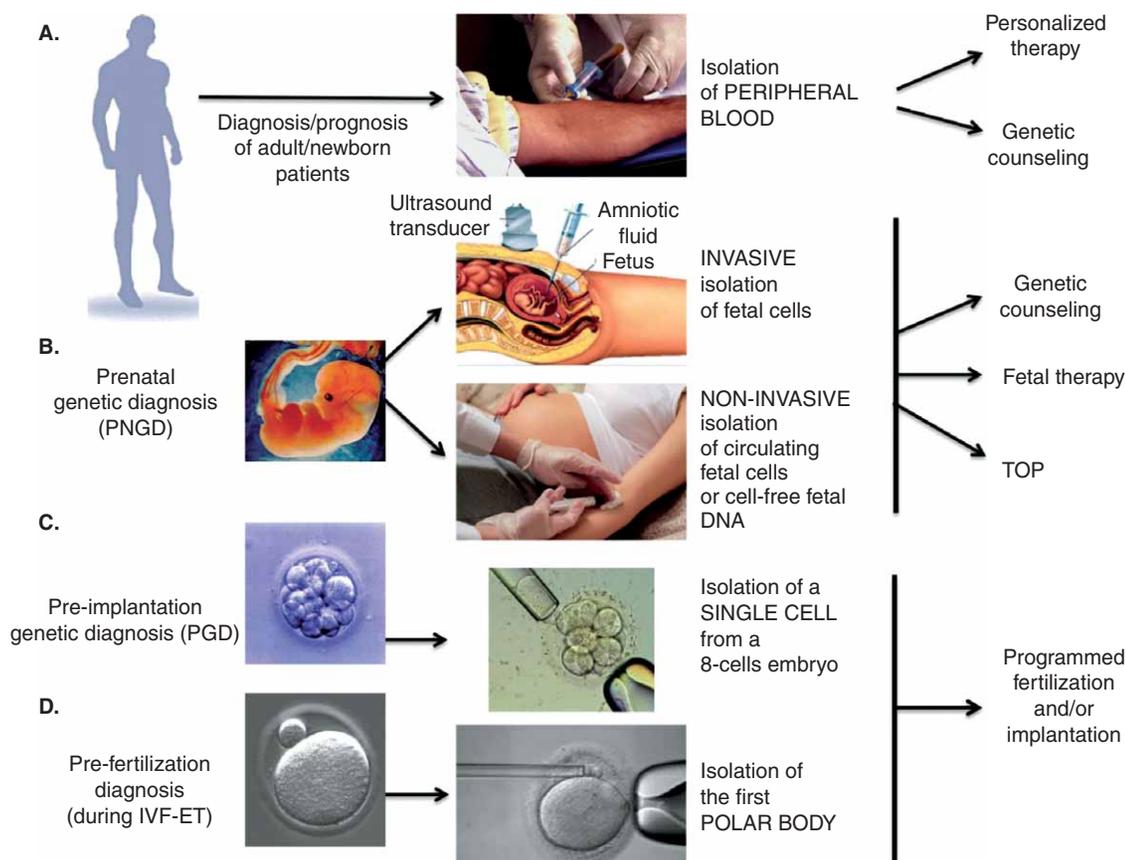


Figure 1. Purposes of genetic analyses performed at different developmental stages. Diagnosis or prognosis on adults or newborns (A), prenatal diagnosis on embryos or fetuses (B), pre-implantation diagnosis on fertilized oocytes (C), pre-fertilization diagnosis on polar bodies (D). For each approach, the required biological material and the possible applications are indicated.

IVF-ET: *In vitro* fertilization – embryo transfer; TOP: Termination of pregnancy.

respectively, as well as a relative increase in γ -globin mRNA levels. Conversely, patients with α -thalassemia showed a decreased ratio of α/β -globin mRNA proportional to the number of deleted α -globin genes. In a second paper, the same group reported that the method of PCR amplification of cDNA copies of circulating erythroid cell mRNA allows us to detect a significant amount (>15%) of normally processed β -globin mRNA, that is produced even in the case of IVSII-654 splicing mutation [19]. The patent associated with this research approach is US5281519 (Title: Simple, rapid and reliable method for detecting thalassemia), which teaches how to reach a diagnosis of thalassemia using this PCR-based approach and determining the ratio between α and β HbA chain mRNAs. In summary, this patent provides an example of a rapid and reliable method, without *in vivo* use of radioactive material, for the diagnosis of thalassemias [20].

Another example of the importance of detecting a possible β -thalassemia is given by the patent application US20120052516 [21]. The inventors made the observation that it is possible to detect and quantify the free α -Hb in

blood samples with efficiency, therefore helping in the fast diagnosis, staging and/or monitoring of HbA-related disorders. Despite the fact that the approach described in this patent should be validated in multiple centers before being widely accepted, the detection of free α -globin chains is clearly of importance, as it is well known that the higher the value of the free α -globin chain pool, the greater the severity of the HbA disorder [1-3,22]. It should be noted that approaches enabling detection of free α -globins may also be useful for identifying new mutations on β -globin genes, as high levels of free α -globin pool are indicative of the presence of β -globin gene mutations, in agreement with the known features of β -thalassemia [22]. Furthermore, this method could be applied to follow therapeutic approaches based on the induction of adult HbA (such as in the case of treatment of β -thalassemia and SCA patients with hydroxyurea), in which a decrease of free α -chains is indicative of the efficacy of the therapeutic treatment.

While the identification of the β -thalassemia phenotype without characterization of the genetic mutation is in any

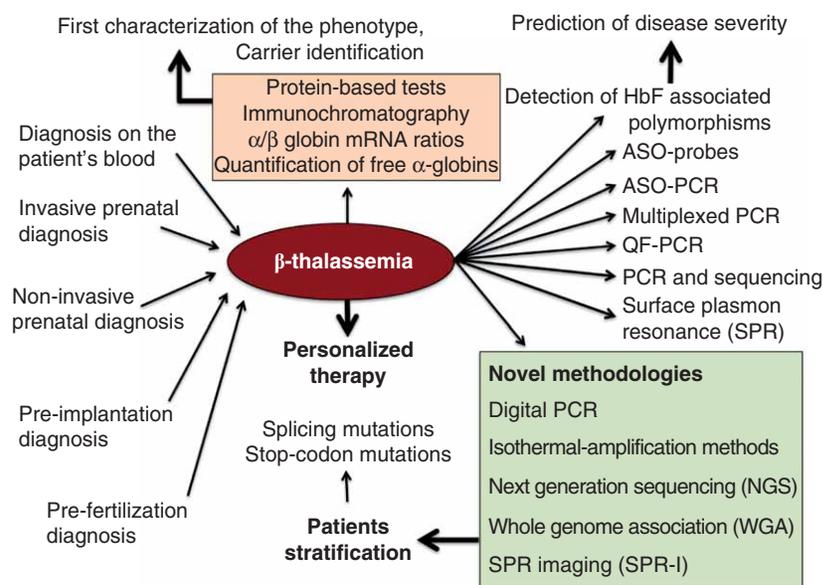


Figure 2. Schematic representation of methods and molecular techniques available for the different diagnostic approaches for β -thalassemia, as well as relative possible outcomes and applications.

PCR: Polymerase chain reaction.

case useful for planning basic therapeutic treatments (i.e., blood transfusions and chelation therapy), the knowledge of the molecular defect causing β -thalassemia is a very useful step for the development of personalized therapies.

For all these specific applications, methods allowing fast, reproducible and efficient detection of single β -thalassemia causing mutations are required and were developed using ASO-probes and Southern blotting, ASO-PCR, multiplexed PCR, in both radioactive and non-radioactive assets. Although all these methods were found useful for detecting specific mutations, they display deep differences with respect to efficiency and sensitivity. Only few of them, therefore, are suitable for diagnosis when limited amounts of biological samples are available (such as in the case of prenatal or pre-implantation diagnostics).

3. Diagnosis of thalassemia: the molecular defect

With respect to key general patents on molecular diagnosis of the genetic basis of β -thalassemia, among the first and the major representative US patents that teach how to efficiently perform this laboratory activity there is US5137806 (Methods and compositions for the detection of sequences in selected DNA molecules) [23] which, as also indicated in Table 1 [17,20,21,23-36], is related to polymerase chain reaction-based procedures and primers for DNA sequence amplification to detect sequence mutations. The PCR primers of the invention incorporate a 3'-terminal nucleotide or nucleotides complementary to the sequence variance, and thereby serve to successfully prime chain elongation only on

DNA templates which include the particular variant (or mutation). This approach was claimed to be useful for molecular diagnosis of thalassemia and SCA.

In consideration of the fact that β -thalassemia is caused by hundreds mutations with different geographic distribution and generating a wide range of severity of the disease also in association with other defects of the globin genes (i.e., association with α -thalassemia) [28,30,36], three important areas of research were followed, that is, i) multiplexing the molecular analysis, ii) development of diagnostic kits selective for specific geographic areas and iii) description of efficient methods for identification of alterations affecting the α -globin gene cluster. Representative examples of patents and patent applications related to these issues are reported in Table 1 [17,20,21,23-36]. For example, the patent application WO1993018178 (Title: Diagnosis of β -thalassemia using a multiplex amplification refractory mutation system) [24] describes a method for characterization of the β -thalassemia gene defect comprising the steps of obtaining genomic DNA from a patient suspected of carrying β -thalassemia and selecting at least two PCR primer sets for detecting at least two mutations characteristic of β -thalassemia. Each primer set was comprised of two primer pairs, a first primer pair comprising a specific primer for a normal allele, and a second primer pair comprising a specific primer for a mutant allele. Each pair further comprised a common primer. This strategy allows to detect multiple mutations within the same PCR reaction tube. As far as molecular diagnosis of α -thalassemia, examples of granted patents are represented by US5750345 (Title: Detection of human α -thalassemia mutations and their use as predictors of blood-related disorders) [28]

Table 1. Partial list of patents and patent applications on diagnosis of the basic molecular gene defects causing β -thalassemia.

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US5137806 [23]	11 Aug 1992	Methods and compositions for the detection of sequences in selected DNA molecules	Anne LeMaistre, Ming-Shen Lee	Board of Regents, the University of Texas System	The patent relates to novel procedures and primers for use with polymerase chain reaction (PCR) or <i>in vitro</i> DNA sequence amplification to detect sequence modifications or mutations. Primers incorporate a 3' terminal nucleotide or nucleotides complementary to the sequence mutation.	Examples of mutations which can be characterized are those present in diseases such as sickle cell anemia (SCA), α - and β -thalassemia.
WO1993018178 [24]	16 Sept 1993	Diagnosis of beta-thalassemia using a multiplex amplification refractory mutation system	Paolo Fortina, Saul Surrey	Philadelphia Children Hospital	The patent describes methods of diagnosis of β -thalassemia effective for simultaneously detecting multiple allelic mutations quickly and accurately.	The methods comprise the steps of obtaining genomic DNA from a patient suspected of carrying β -thalassemia and selecting at least two PCR primer sets for detecting at least two β -thalassemia mutations.
US5328824 [25] Published also as: US4711955 US5449767 US5476928	12 Jul 1994	Methods of using labeled nucleotides	David C. Ward, Pennina R. Langer, Alexander A. Waldrop III	Yale University	DNA analogues are described capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid.	Some uses include detecting and identifying genetic disorders, for example, thalassemia and SCA. Detection and localization of polynucleotide sequences is possible in chromosomes, fixed cells, tissue sections and cell extracts.
US5281519 [20] Published also as: WO1991015592	25 Jan 1994	Simple, rapid and reliable method for detecting thalassemia	Alan N. Schechter, Shu-Zhen Huang, Griffin P. Rodgers	Department of Health and Human Services, USA	A simple, rapid and reliable method for diagnosis of thalassemia. The method comprises amplification of the cDNA by PCR and determination of the ratio between α - and β -globin mRNAs.	The patent provides an example of a rapid and reliable method, without <i>in vivo</i> use of radioactive material, for the diagnosis of thalassemias. The method was proposed for planning treatment, prevention and genetic counseling.
US5312527 [26]	17 May 1994	Voltammetric sequence-selective sensor for target polynucleotide sequences	Susan R. Mikkelsen, Kelly M. Millan, Aleksandris J. Spurmanis	Concordia University	The present invention relates to a voltammetric sequence-selective sensor for target polynucleotide sequences which essentially comprises an immobilized polynucleotide probe having one end covalently bound onto an amperometric electrode.	The main aim of the present invention is to provide a sequence-selective sensor capable of precisely detecting target polynucleotide sequences, enabling the diagnosis of inherited diseases such as sickle cell anemia and β -thalassemia.

Table 1. Partial list of patents and patent applications on diagnosis of the basic molecular gene defects causing β -thalassaemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US5747256 [27] Published also as: CA223961 EP0868528 WO1997022718	5 May 1998	Homogeneous DNA probe titration assay	Cheng F. Yan, Fredrick S. Yein	Beckman Instruments, Inc.	The assay described comprises the steps of forming a reaction mixture by combining (i) a first reagent and (ii) an aliquot of the test sample suspected of containing the target DNA sequence. After addition of a second reagent, the change in turbidity of the reaction mixture correlates with the presence of the target polynucleotide in the sample.	Described by the patent is an assay for detecting the presence or amount of a target DNA sequence of interest in a test sample. The assay can be performed on test samples such as serum, plasma, saliva, cerebral spinal fluid, amniotic fluid, urine, feces, mucus, cell extracts, tissue extracts and pus.
US5750345 [28] Published also as: EP0859863 WO1997016568	12 May 1998	Detection of human α -thalassaemia mutations and their use as predictors of blood-related disorders	Lemuel J. Bowie	Evanston Hospital Corporation	The invention provides an improved method for determining a human subject's genotype at the α -gene loci. Furthermore, the invention provides an apparatus/kit for screening a human subject for a risk of developing blood-related disorders.	The genetic marker can be used alone or in combination with other genetic markers for predicting the development of a blood-related disorder with respect to a particular gene locus (i.e., the α -globin gene locus on human chromosome 16).
US5958692 [29] Published also as: CA2188660 DE69531612 EP0760002 US5698400 WO1995029251	28 Sep 1999	Detection of mutation by resolvase cleavage	Richard G. H. Cotton, Rima Youil, Borries W. Kemper	Variagenics, Inc.	Methods are disclosed for detecting one or more mutations in an isolated test nucleic acid by forming a heteroduplex with a homologous control DNA and contacting the heteroduplex with a resolvase capable of recognizing at least one single base pair mismatch within the heteroduplex.	In general, the invention features a method for detecting one or more mutations in an isolated test nucleic acid which preferentially hybridizes to an isolated control DNA. It may be useful to detect pathogenic mutations responsible for genetic diseases.
US6322981 [30] Published also as: WO1998023778	27 Nov 2001	Rapid method for diagnosing the various forms of alpha-thalassaemia	Griffin P. Rodgers, Delia C. Tang	Department of Health and Human Services, USA	The present invention relates to the simultaneous and specific identification of the variant forms of α -thalassaemia. The approach is based on a simple and rapid non-radioisotopic technique for the diagnosis and differentiation of the common forms of α -thalassaemia.	The diagnostic strategy described in the patent works on any biological tissues, including blood. The assay works equally well with fresh blood and dried blood samples stored on filter paper.

Table 1. Partial list of patents and patent applications on diagnosis of the basic molecular gene defects causing β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US6777183 [31] Published also as: CA2405687 EP1320628 US20020009716 WO2001077390 WO2001077390	17 Aug 2004	Process for allele discrimination utilizing primer extension	Patricio Abartzúa	Molecular Staging, Inc.	This patent describes methods for allele discrimination involving the use of rolling circle amplification (RCA) coupled with primer extension and utilizing exonuclease deficient polymerases to distinguish matched and unmatched single nucleotide sites, such as in the case of a single nucleotide polymorphism (SNP).	The purpose of this approach is to provide a simple and ready mean of genotyping using the ability of a probe to detect mismatches in a target polynucleotide sequence. It may be very useful in detecting single nucleotide polymorphisms (SNPs), as well as other mutations, and for use in molecular haplotyping.
WO2005078132 [32] Published also as: WO20070161032	25 Aug 2005	Oligonucleotide probes for diagnosis and identification of the different forms of β -thalassemia, methods and diagnostic kits thereof	Antonino Giambona, Aurelio Maggio	Biochemical Systems Internaton, Antonino Giambona, Aurelio Maggio	The method is based on Reverse Dot Blot (RDB) assay, wherein, after hybridization, a colorimetric reaction and a subsequent formation of an insoluble precipitate take place.	The invention relates to oligonucleotide probes for identification and diagnosis of gene mutations associated with β -thalassemia.
US20070161032 [33] Published also as: CN1982477 EP1798291	12 Jul 2007	Methods and compositions for assaying mutations and/or large scale alterations in nucleic acids and their uses in diagnosis of genetic diseases and cancers	Jean-Louis Viowy, Claude Houdayer, Dominique Stoppa-Lyonnet, Jeremie Weber	Institut Curie, Centre National De La Recherche Scientifique	The method described is based on the study of homoduplexes and possible heteroduplexes, conducting on said products an analytical method suitable for obtaining at least signal(s) discriminating the existing duplex form(s) of the first nucleic acid fragment and relative quantitative data.	It is the main object of the invention to allow the simultaneous search of large scale and local mutations, and thus a simplification and an acceleration of the mutation search effort.
EP1816215A1 [34] Published also as: WO2007089145	8 Aug 2007	Disease specific ASO-probes for the detection of alpha- and beta-thalassemia mutations	Cornelia Hillegonda Van Moorsel, Cornelis Leonard Harteveld, Johannes Theodorus Den Dunnen, Piero Carlo Giordano	Academisch, Ziekenhuis Leiden	Allele Specific Oligo-probes (ASO-probes) were designed for the large scale high-throughput detection of in total 66 β - and 42 α -thalassemia mutations. Oligo-probes were designed as 20- or 21-mers for which the SNP-position was located in the middle of the probe (position 0).	A collection of probes covering many mutations causing α - and β -thalassemia worldwide is provided, applicable to the diagnosis of thalassemia in patients from many countries in which thalassemia is endemic and causes a serious health problem. Moreover, post- and neonatal screening is feasible, together with the determination of the risk or the increased risk of a child to develop thalassemia.

Table 1. Partial list of patents and patent applications on diagnosis of the basic molecular gene defects causing β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US20120052516 [21] Published also as: EP2422203 WO21010122160	1 Mar 2012	Method for diagnosing a hemoglobin-related disorder	Veronique Baudin-Creuz, Corinne Vasseur, Frederic Galacteros	Veronique Baudin-Creuz, Corinne Vasseur, Frederic Galacteros	The invention relates to a method for diagnosing, staging and/or monitoring a HbA-related disorder such as β -thalassemia or a treatment against said HbA-related disorder in a subject in need thereof based on the detection and/or quantification of free α -Hb pool in a biological sample obtained from the subject.	The possible detection and quantitation of the free α -Hb in a blood sample is useful for diagnosing, staging and/or monitoring HbA-related disorders. Higher is the value of the free α -Hb pool, more the HbA disorder is severe. The method may also be useful for identifying new mutations on globin genes, as high levels of free α -Hb pool are indicative of the presence of mutations.
US8097405 [17] Published also as: CA1223831 DE338262 DE338278 DE338282 EP0097373 EP0285057 EP0285058 EP0285950 EP0286898 EP0302175 EP0618228 US6992180	17 Jan 2012	Nucleic acid sequencing processes using non-radioactive detectable modified or labeled nucleotides or nucleotide analogs, and other processes for nucleic acid detection and chromosomal characterization using such non-radioactive detectable modified or labeled nucleotides or nucleotide analogs	Dean Engelhardt, Elazar Rabbani, Stanley Kline, Jannis G. Stavrianopoulos, Dollie Kirtikar	Enzo Biochem, Inc.	The patent describes a method for determining the sequence of nucleic acids of interest using nucleotides or nucleotide analogs that have been made detectable by non-radioactive modifying or labeling. Such nucleotides or nucleotide analogs are modified on the sugar moieties, the phosphate moieties or the base moieties, including base analogs.	This invention allows to detect mutated DNA sequences in inherited genetic diseases.
WO2012137110 [35] Published also as: CN103649332 EP2694674 US20140148344	11 Oct 2012	Association markers for beta thalassemia trait	Nevenka Dimitrova, Sina Vivekanandan Kadavil, Sunil Kumar, Randeep Singh	Koninklijke Philips Electronics N.V.	The strategy comprises the steps of: (a) isolating a nucleic acid from a subject's sample, (b) determining the nucleotide sequence and/or molecular structure present at one or more of the mentioned polymorphic sites, wherein the presence of an indicator nucleotide is indicative of the presence of β -thalassemia.	This invention addresses the need for an easier, more straightforward, more sensitive and more specific strategy for screening and detection of β -thalassemia, providing means and methods which allow the detection and identification of this disease, in particular of β -thalassemia carriers.

Table 1. Partial list of patents and patent applications on diagnosis of the basic molecular gene defects causing β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US8563330 [36] Published also as: EP1972941 US20080233659	22 Oct 2013	Process of screening for alpha-thalassemia carrier using immuno chromatographic strip test	Watchara Kasinrerk, Chatchai Tayapiwatana, Thanusak Tatu, Suthat Fucharoen, Sawitree Chiampanichayakul	National Science and Technology Development Agency	The patent provides the description of a device and method for the rapid identification of patients suspected of having thalassemia. The invention provides a test strip for the aqueous detection of thalassemia related proteins in whole blood. The invention can be used in remote or clinical settings.	The immunochromatographic strip test presented in this invention is recommended for the detection of Hb Bart's protein in a human whole blood specimen. The immunochromatographic strip test shows positive reading for α thal 1 trait, HbH (α thal1/ α thal2), HbH-CS (α thal1/Hb CS), AEBart's (HbH disease + HbE trait), Hb Bart's hydrops fetalis (homozygous α thal1) and homozygous α thal 2.

and US6322981 (Title: Rapid method for diagnosing the various forms of α -thalassemia) [30]. In consideration of the importance to obtain information for several mutations causative of β -thalassemia and, at the same time, detecting α -thalassemia, the patent EP1816215 (Title: Disease specific ASO-probes for the detection of α - and β -thalassemia mutations) [34] should be mentioned. In this patent, oligonucleotide-probes were designed for the detection of in total 66 β - and 42 α -thalassemia mutations. The described Allele Specific Oligonucleotides for hybridization (ASO-probes) were designed as 20- or 21-mers for which the SNP-position was located in the middle of the probe. This set of ASO-probes can be used for large-scale high-throughput post- and neonatal screening programs aimed at prevention of these severe recessive disorders.

4. Molecular diagnosis of thalassemia and personalized therapy

Personalized therapy is one of the major issues in molecular medicine [11,37]. Examples of personalized medicine involve the choice of the therapeutic drug after considering the genotype (in terms of analysis of the basic genetic mutations as well as of the wide-genome polymorphism scanning) [38] and the phenotype (in terms of transcriptomic and proteomic pattern) [39]. In addition, the knowledge of the molecular basis of the diseases is expected to help in the development of disease-specific *in vivo* models as well as disease-specific therapeutic approaches. This issue is very complex in consideration of the fact that even monogenic diseases can be caused by a large variety of genetic mutations. In the case of β -thalassemia, this area of intervention is very important, but considering limitations and side effects of the therapeutic approaches and management of the thalassemia patients, novel alternative options for therapy are urgently needed, based on the genetic background of the patient. These approaches deserve great attention and should be considered together with other very promising therapeutic strategies, such as gene therapy and genome editing using Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Nucleases (TALENs) or the Clustered Regularly Interspaced Short Palindromic Repeat/Protein-9 Nuclease (CRISPR/Cas9) system [3].

4.1 Molecular approaches for a personalized therapy of β -thalassemia: mutations affecting splicing

A very exciting possibility linking diagnostics to therapeutic choices is the study by Svasti *et al.*, who described a very interesting approach finalized to the repair of β -globin pre-mRNA rendered defective by a thalassemia-causing splicing mutation, IVSII-654, in intron 2 of the human β -globin gene [40]. This intervention was performed using a mouse model of IVSII-654 thalassemia, and based on the use of the delivery of a splice-switching oligonucleotide (SSO), a morpholino oligomer conjugated to an arginine-rich peptide. The SSO

blocked the aberrant splice site in the target pre-mRNA and forced the splicing machinery to reselect existing correct splice sites. Repaired β -globin mRNA restored significant amounts of HbA in the peripheral blood of the IVSII-654 mouse, improving the number and quality of erythroid cells. This approach is expected to be used in all the several splicing defects of β -thalassemia (although they do not represent the majority of the β -thalassemia mutations) which produce large amounts of incorrectly spliced RNA molecules deeply interfering with RNA trafficking and translation. Despite the fact that major limitations still exist, including efficient delivery of the SSO to target cells [3], these studies represent new hopes for specific classes of β -thalassemia patients carrying splicing mutations.

4.2 Molecular approaches for a personalized therapy of β -thalassemia: stop-codon mutations

Another example of the need of genomic characterization of β -thalassemia patients is related to the recently proposed read-through approaches for inducing HbA production in erythroid precursors from patients affected by $\beta^{0/39}$ -thalassemia, where the CAG (glutamine) codon is mutated to the UAG stop codon, leading to premature translation termination and to mRNA destabilization through the well-described NMD (nonsense-mediated mRNA decay) [41]. Relevant to this issue, Salvatori *et al.*, after FACS and HPLC analyses, demonstrated that erythroid precursor cells from $\beta^{0/39}$ -thalassemia patients are able to produce β -globin and adult HbA after treatment with G418 [42]. This study strongly suggests that ribosomal read-through should be considered a strategy for developing experimental approaches for treatment of β^{0} -thalassemia caused by stop codon mutations and might be combined with DNA-based strategies to reactivate HbF production [42]. Accordingly, the identification of patients carrying stop-codon mutations might be relevant to design for them a therapeutic intervention based on the use of read-through molecules. This approach, even if not used yet in clinical trials of β -thalassemia, should be considered as it is followed also for other genetic diseases caused by nonsense mutations, such as cystic fibrosis [42].

5. Diagnosis of thalassemia polymorphisms associated with fetal HbA production

This issue has been the object of several studies in the field of β -thalassemia, well discussed in a paper by Thein and Menzel [43], reporting the progress in the understanding of the persistence of HbF in adults. Three major loci (XmnI-HBG2 single nucleotide polymorphism, HBS1L-MYB intergenic region on chromosome 6q, and BCL11A) contribute to high HbF production. Although other numerous loci are expected to be present [44,45] and other genes involved in HbF production (such as for example KLF1 and Oct-1), further research efforts are needed to verify their relevance

and specific effects. It should be pointed out that the identification of the three major loci has not yet been translated into new therapeutic approaches for HbF reactivation but, in consideration of the fact that they might identify putative binding sites for transcription repressors, an immediate application is expected to be the prediction of the ability of erythroid cells carrying such genetic variations to produce HbF, which in turn may improve prediction of disease severity and possible personalized therapeutic strategies [43].

The key patent concerning this specific issue is EP2185733 (also reported as US20100216664, and WO2009007685) [46], published on May 19, 2010 and entitled "Method and markers for determining the severity of β -hemoglobinopathies" (inventors: Mark Lathrop and Swee Lay Thein, original assignees: King's College London, Commissariat à l'Energie Atomique). As stated in the description of the patent, the invention relates to a method for determining the severity of a disease attributed to genetic mutations in one or more globin genes. In the method the diagnostic steps comprise: i) providing a blood sample for genomic DNA isolation and ii) determining the presence of one or more diagnostic markers. The described markers are located: i) within a 127 kb segment on chromosome 2p15; ii) within MYB and/or HBSIL and/or the intergenic region between MYB and HBSIL located on the 6q23 QTL interval; and/or iii) within one of several chromosomal loci. This strategy, therefore, will allow us to stratify the patients with respect to several polymorphic loci; the most relevant are those concerning MYB, BCL11A and the XmnI polymorphism. As commented in the description of the patent, the presence of said marker(s) in the sample is indicative that the disease will be less severe in those affected subjects in comparison to a subject that does not possess the marker(s).

As far as the several publications supporting the translational applications of the approach described in patent EP2185733, few examples are those published by Badens *et al.* [47], Danjou *et al.* [38] and by Banan *et al.* [48]. Badens *et al.* genotyped >100 patients for 5 genetic modifiers of severity: i) β -thalassemia mutations; ii) the XmnI SNP; iii) the -3.7 kb α -thal deletion; iv) the tag-SNP rs 11886868 in BCL11A exon 2; and v) the tag-SNP rs9399137 in the HBSB1L-cMYB inter-region [47]. Multivariate analysis was performed to study the risk of thalassemia intermedia phenotype associated with the different combinations of alleles. They showed that predictions based on genetic modifiers can foresee the major or intermedia type of β -thalassemia, even in cohorts of patients with various β -globin genotypes [47]. Danjou *et al.* characterized several genetic variants at the XmnI, HBS1L-MYB intergenic region and BCL11A loci robustly associated with the amelioration of β -thalassemia phenotype, to build a predictive score of severity using a representative cohort of 890 β -thalassemic patients [38]. Using Cox proportional hazard analysis on a training set, they assessed the effect of these loci on the age at which patients started regular transfusions, and validation was conducted on the

testing set confirming its predictive accuracy with transfusion-free survival probability and with transfusion dependency status [38]. Finally, Banan *et al.* found that the presence of the specific XmnI genotypes or the BCL11A rs766432 C allele strongly correlates with response to hydroxyurea, measured as the increase in blood transfusion intervals post-therapy [48]. Although this observation needs to be validated in other cohorts of patients, these markers can be proposed to verify the possibility to predict hydroxyurea response.

6. Non-invasive prenatal diagnosis

In Table 2 examples are listed of patents dealing with prenatal genetic diagnosis (PNGD), including invasive prenatal diagnosis, non-invasive prenatal genetic diagnosis (NIPNGD), pre-implantation genetic diagnosis (PGD) and pre-conception molecular diagnosis, as well as methods used for the optimization of these approaches [49-75].

The issue of prenatal diagnosis faces with the fact that it often requires invasive testing by amniocentesis, chorionic villus biopsy or fetal blood sampling [8]. These diagnostic techniques increase the frequency of fetal loss by ~0.5%. One alternative way for obtaining information on the gestating fetus involves recovery of fetal cells from maternal blood [76-78]. In this respect, trophoblast cells have not found widespread application in diagnostic studies because they are rapidly cleared by the maternal pulmonary circulation and are likely to exhibit confined chromosomal mosaicism. On the contrary, nucleated red blood cells (NRBC) are the most common cells in fetal blood during early pregnancy [76,78]. Because they have a relatively short half-life, and because they express hematopoietic plasma membrane antigens, such as the transferrin receptor (CD71), the glycophorin A cell surface molecule and intra-cellular markers (ϵ - and γ -globin chains), fetal NRBC have become the targets of choice. With respect to analytic molecular biology techniques, the most important molecular approaches that have allowed genetic analysis of enriched fetal cells are PCR and FISH. The ability of PCR to amplify minute quantities of DNA (even single copies) over a billion fold has been exploited for the prenatal diagnosis of monogenic disorders from maternal blood. Up to date, all of these methods result in the enrichment of fetal cells among larger populations of maternal cells, but they do not enable recovery of pure populations of fetal cells. Experimental approaches that combine fetal cell identification with molecular genetic diagnosis in an *in situ* technique circumvent these limitations and are especially suited for automation.

As far as the key patents in this specific field of investigation, US5405751 (Title: Prenatal diagnosis by cytokine-induced proliferation of fetal T-cells) [49] teaches how to propagate fetal T-cells co-isolated from amniotic fluid using a cytokine cocktail. Different protocols for the identification and enrichment of the biological sample with fetal cells have also been described

in US5641628 [50], US5750339 [51], US7651838 [58] and the patent application WO2009009769 [56].

On the other hand, with the advent of the concept that circulating cell-free RNA and DNA molecules are present in the peripheral blood of pregnant women [79,80], non-invasive prenatal diagnosis has been based on the study of circulating free-DNA (Figure 3). In this respect, several patents deal with methods for enriching circulating free-DNA of fetal-type DNA. This step is key to obtain robust data even in the early phases of the gestation. Examples of patents and patent applications dealing with this specific issue are reported in Table 2. For example, patent EP1664077 (Title: Method for non-invasive prenatal diagnosis) [53] describes a method based on a novel combination of a base extension reaction, which provides excellent analytical specificity, and a mass spectrometric analysis, which provides excellent specificity for nucleic acid detection. The method presented allows accurate detection of nucleic acids that are present in very small amounts in a biological sample, including fetal nucleic acid detection and analysis in maternal blood, plasma and serum samples. The non-invasive prenatal diagnosis has a very solid scientific background as demonstrated by several studies [9,81], many of which also focusing on non-invasive prenatal diagnosis of β -thalassemia and SCA [82-85].

7. Novel technologies and trends in diagnostics

In order to identify specific DNA sequences and to find pathogenic point mutations, DNA analyses are required, such as the currently used comparative genomic hybridization (CGH) [86], PCR and surface plasmon resonance (SPR) analysis [87], quantitative fluorescent (QF) PCR [88]. Novel technologies are available for diagnostics as well, including multiplex ligation-dependent probe amplification (MLPA) [89,90], digital PCR [91] and next-generation sequencing (NGS) [92,93]. MLPA is a multiplex PCR-based assay that allows us to determine the copy numbers of up to 50 different genomic DNA sequences in only a single reaction tube [89]. In prenatal diagnosis it is considered a quick, clear, inexpensive genetic screening method for detection of aneuploidies in pregnancies at high risk [90]. Digital PCR, first developed in 1999, is based on limiting dilution of DNA and single molecule detection to identify and quantify the target mutated DNA [91]. Only a small amount of template is required for analysis, so it can be very useful for identifying rare variants or in non-invasive prenatal diagnosis, where it has been successfully used for the detection of hemoglobinopathies [85].

NGS, which was introduced in 2005, indicates a high high-throughput DNA sequencing technology capable to analyze large amounts of different DNA samples in a single reaction [92]. Different commercial platforms are currently available for this technology: Genome Analyzer and HiSeq 2000 (Illumina), HeliScope (Helicos BioSciences), SOLiD and Ion Torrent (Life Technologies), Roche/454 (Roche).

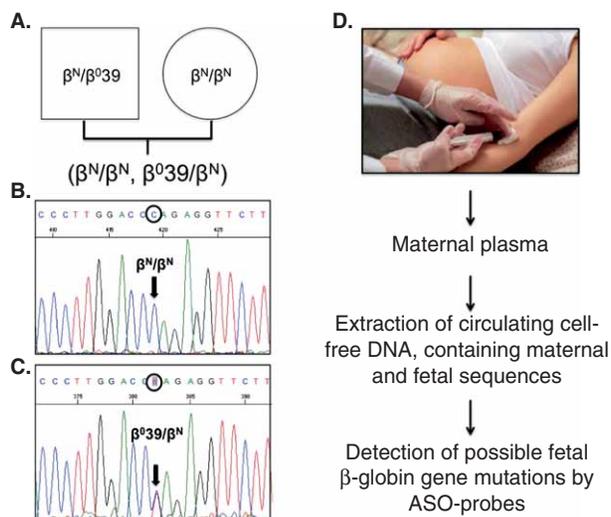


Figure 3. Example of non-invasive prenatal diagnosis of β -thalassemia point mutations inherited from the father. In this example, the father is a heterozygous carrier for the β^{39} mutation, whereas the mother does not carry the alteration on her β -globin genes, resulting in two possible fetal genotypes (A). (B,C) Portions of electropherograms obtained after sequencing the β -globin genes of the β^N/β^N mother (B) and the β^{39}/β^N father (C), respectively. (D) Non-invasive experimental strategy used to detect paternally inherited β -globin gene mutations in the fetus, only requiring a peripheral blood sample from the pregnant mother.

In all of them, either templates, primers or polymerase enzymes are immobilized on a solid support or on beads before the sequencing reactions, allowing the instruments to process millions of microreactions carried out in parallel on each spatially distinct template. Anyway, the final step of NGS is genome alignment, performed through several strategies for filtering and scoring the different variants and for detecting disease-causing mutations [92]. Thanks to the advent of NGS, resolving the whole genome of a fetus from maternal blood sources has become possible, demonstrating that these technologies can be applied in prenatal diagnosis for detection of severe monogenic disorders, fetal chromosomal aneuploidies and blood groups [93].

Examples of patents and patent applications on the described technologies are EP1130113 (Title: Multiplex ligation dependent amplification assay) [74], which describes biomedical applications of MLPA and US8722334 (Title: Analysis for nucleic acids by digital PCR) [75], which describes a method for analyzing nucleic acids for their lengths and relative abundance in a sample, based on digital amplification of individual template molecules. A comprehensive review of patents on the use of NGS for molecular diagnosis has been recently published by Lin *et al.* [94].

Finally, several articles have been published dealing with PCR-free methods for detection of point mutations. In fact, although PCR is a simple, sensitive, and cost-effective technology, it is prone to sample contamination and suffers from biases in the template-to-product ratios of the amplified target sequences [95]. The need for repeated heating and cooling steps is an important limitation of the method, especially in the case of the full exploitation of advantages associated with the integration of nucleic acid amplification procedures in microfluidic-based devices [96]. To overcome this limitation, several alternative isothermal-amplification methods have been developed [97] which do not require thermal cycling, including nucleic acid sequence-based polymerization (NASBA), loop-mediated amplification (LAMP), helicase-dependent amplification (HDA), rolling-circle amplification (RCA), recombinase-polymerase amplification (RPA) and multiple-displacement amplification (MDA). Recently, isothermal circular-strand-displacement polymerization (ICSDP) has emerged as a new and promising method for nucleic acid amplification and detection [96-98]. Finally the direct detection of β -thalassemia mutations in non-PCR-amplified human genomic DNA has been recently demonstrated by surface plasmon resonance imaging (SPR-I). Attomolar concentrations of target genomic DNA were detected from healthy individuals and homozygous or heterozygous patients affected by β -thalassemia, representing an important advantage in several biomedical applications, including prenatal diagnosis [99].

8. Expert opinion

The most recent findings in the diagnostic approach for β -thalassemias are related to three major fields of investigation: i) moving the efficiency of the detection of the primary mutation causative of β -thalassemia to ultrasensitive methodologies, including the development of PCR-free approaches; ii) extension of the genotypic analysis of β -thalassemia patients to high-HbF associated polymorphisms; iii) introducing whole genome association (WGA) assays and next-generation sequencing. The results obtained within this field of investigation should be discussed in the context of several aspects, including regulatory, ethical and social issues.

For example, the European Charter of Patients' Rights states that every individual has the right to a proper service in order to prevent illness. This is clearly linked on one hand to prenatal diagnosis, and on the other hand to early diagnosis helping to propose a personalized therapy [100].

As it was already stated, β -thalassemias are caused by >200 mutations of the human β -globin gene. This issue should be considered together with the suggestion that, whereas for some mutations a possible therapeutic approach is expected, for some of them the cure appears to be far away. In this framework, the patients have the right to know their genotype, and the parents should be informed of the fetus' genotype even prenatally. In this context the issue of

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia.

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
<i>Methods related to prenatal, pre-implantation and pre-fertilization diagnosis</i> US5405751 [49] Published also as: US5879937	11 Apr 1995	Prenatal diagnosis by cytokine-induced proliferation of fetal T-cells	Maria-Grazia Roncarolo	Schering Corporation	A volume of amniotic fluid containing fetal cells is extracted from a pregnant woman, and the fetal T-cells are propagated in a culture medium comprising one or more cytokines. The fetal T-cells are then diagnosed for genetic defects.	Improved methods of prenatal diagnosis are described that can be performed earlier in pregnancy, and/or with greater safety, and/or rapidly, and/or with greater accuracy than conventional methods. A large number of abnormalities and diseases can be diagnosed, comprising hematological disorders, including thalassemia.
US5641628 [50] Published also as: US20020006621 US20020137088 US20040018509 US20060051775 US20070015171 US20080182261	24 Jun 1997	Non-invasive method for isolation and detection of fetal DNA	Diana W. Bianchi	Children's Medical Center Corporation	A method of detecting the presence or absence of a fetal DNA sequence of interest in fetal DNA derived from a sample of peripheral blood obtained from a pregnant woman is described. The proportion of fetal nucleated cells can be increased forming a sample enriched in fetal nucleated cells prior to the detection step.	The present invention relates to an <i>in vitro</i> method of separating or isolating fetal nucleated cells present in the blood of a pregnant woman (a maternal blood sample) from the pregnant woman's cells and of separating or isolating fetal DNA from maternal DNA. The method provides a non-invasive approach to detect a disease, based on the presence or absence of hybridization between the DNA probe and the fetal DNA.
US5750339 [51] Published also as: WO1996017085	12 May 1998	Methods for identifying fetal cells	J. Bruce Smith	Thomas Jefferson University	The patent describes a method for distinguishing fetal cells from maternal cells using a probe complementary to HLA-G (human leukocyte antigen G) mRNA and identifying individual cells in which the probe hybridizes to mRNA in the cells. Absence of hybridization indicates that the cells are maternal cells. Presence of hybridization indicates that the cells are fetal cells.	Fetal cells identified in accordance with the invention may be tested for genetic abnormalities. The invention features a method of distinguishing fetal cells from maternal ones, allowing to detect genetic abnormalities in a fetus, such as β -thalassemia and sickle cell anemia (SCA).
US6258540 [52] Published also as: US20010051341 CA2282793 DE69814639 EP0994963 WO1998039474	10 Jul 2001	Non-invasive prenatal diagnosis	Yuk-Ming Dennis Lo, James Stephen Wainscoat	Isis Innovation Limited	The invention relates to a detection method performed on a maternal serum or plasma sample from a pregnant female, and detects the presence of a nucleic acid of fetal origin in the sample.	The invention enables non-invasive prenatal diagnosis including for example sex determination, blood typing and other genotyping.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
EP1664077 [53] Published also as: EP1664077 EP2354253 US20070207466 US20120225798 WO2005023091	7 Jun 2006	Method for non-invasive prenatal diagnosis	Charles R. Cantor, Chunming Ding, Yuk Ming Dennis Lo, Rossa Wai- Kwun Chiu	The Trustees of Boston University, The Chinese University of Hong Kong	The method is based on a novel combination of a base extension reaction and a mass spectrometric analysis, both providing excellent analytical specificity. The method allows accurate detection of nucleic acids that are present in very small amounts in a biological sample. For example, the method of the present invention is preferably used to detect fetal nucleic acids in maternal blood samples.	This method allows to detect a genetic disease or characteristic in a fetus using maternally blood, plasma, serum. The determination is performed using primers corresponding to the disease-causing mutation and performing an SNP analysis. The amplification of the nucleic acids in the sample employs PCR primers designed to anneal to regions flanking a genetic locus carrying a difference between the maternal and the paternal nucleic acid, performing a base extension reaction.
WO2007028155 [54] Published also as: US20070122823 WO2007028155	8 Mar 2007	Fragment size distribution pattern of amniotic fluid cell-free fetal DNA for prenatal diagnosis	Diana W. Bianchi, Kirby L. Johnson, Olav Lapaire	New England Medical Center Inc, Diana W. Bianchi, Kirby L. Johnson, Olav Lapaire	The patent includes analysis of the fragment size distribution of cell-free fetal DNA from amniotic fluid. The method is rapid and does not require cell culture.	The inventive methods allow for rapid screening of fetal characteristics such as chromosomal abnormalities and for prenatal diagnosis of a variety of diseases and conditions.
WO2008019315 [55] Published also as: US20080182244 WO2008019315	14 Feb 2008	Improved pre-implantation genetic diagnosis test	Triantafyllos Tafas, Iliia Ichetovkin, Michael Kilpatrick, Petros Tsipouras	Ikonisys Inc, Triantafyllos Tafas, Iliia Ichetovkin, Michael Kilpatrick, Petros Tsipouras	A method for determining viable normal blastomeres for implantation entailing labeling the blastomere with an antibody to hyperglycosylated hCG and determining the binding of chromosomal probes directed to chromosomal regions is described.	PGD is particularly useful for couples with a known history of genetic diseases. This early screening of embryos allows only embryos without known genetic defects to be returned to the uterus.
WO2009009769 [56] Published also as: WO2009009769 US20090181421	9 Apr 2009	Diagnosis of fetal abnormalities using nucleated red blood cells	Ravi Kapur, Diana Bianchi, Tom Barber, Mehmet Toner	Artemis Health Inc, Gen Hospital Corp, Tufts Medical Ct Inc, Gpb Scient Llc, Ravi Kapur, Diana Bianchi, Tom Barber, Mehmet Toner	The present invention relates to methods for diagnosing a condition in a fetus by enriching and enumerating circulating red blood cells with the possible combination of results from maternal serum marker screens.	The presence of fetal cells in the peripheral blood of a pregnant mammal provides an opportunity to practice prenatal diagnostics without the risks associated with more invasive diagnostic procedures. Enrichment of fetal cells from peripheral blood facilitates the analysis of these cells and makes it easier to diagnose fetal abnormalities.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US7713728 [57] Published also as: US20080166714	11 May 2010	Screening polar bodies for genetic defects	W. Daniel Hillis, Roderick A. Hyde, Edward K. Y. Jung, Robert Langer, Nathan P. Myhrvold, Lowell L. Wood JR.	The Invention Sciencefund I, Llc	The present application relates to methods of selecting germ line genomes at least partially based on one or more genetic characteristics of the germ line genomes and related systems implementations, apparatus and/or compositions. Germ line genomes may be selected to include certain target genetic characteristics and/or to exclude certain target characteristics as optionally determined by a system operator.	The proposed strategies for selecting female germ line genomes are based on selection methods and/or polar body subtractive determination-based selection methods. As used herein, the term 'related polar bodies' means one or more of the first and second polar bodies that arise during meiosis of a primary oocyte. The three polar bodies that arise from single primary oocyte are 'related' as used herein.
US7651838 [58] Published also as: CA2452099 EP1383926 US20050049793 WO2002088736	26 Jan 2010	Prenatal diagnosis method on isolated foetal cell of maternal blood	Patrizia Paterlini-Brechot	Institut National De La Sante Et De La Recherche Medicale, Assistance Publique-Hopitaux De Paris, Universite Rene Descartes Paris 5	The invention concerns a novel non-invasive prenatal diagnostic method implemented with a sample of maternal blood. Said method enables prenatal diagnosis on isolated non-apoptotic epithelial fetal cells of maternal blood after they have been enriched by filtration, morphologically or immunologically and genetically analyzed.	The described method allows prenatal diagnosis to be carried out on non-apoptotic epithelial fetal cells isolated from maternal blood after enriching them by filtration, and their morphological or immunological and genetic analysis. Advantages of the method are its harmlessness towards mother and fetus and very high sensitivity and specificity of the diagnosis.
<i>Novel methodologies</i> EP0154505 [59] Published also as: DE3586689 EP0154505	30 Sep 1992	Diagnosis of gene abnormalities by restriction mapping using a sandwich hybridization format	John E. Monahan, Stephen H.C. Ip, Charles Rittershaus	Ortho Diagnostic Systems Inc.	Double stranded sample nucleic acid is reacted with a restriction endonuclease whose activity depends on the presence or absence of the particular base sequence to be detected at the restriction site. The DNA is denatured and reacted with an immobilized nucleic acid probe and a labeled nucleic acid probe each of which hybridizes on either side of the restriction site. Separation of the aqueous and solid phases and measurement of the label relates to the presence or absence at the restriction site of the particular base sequence.	In accordance with the principles and objects of the present invention there are provided methods for determining the presence of specific genetic sequences in sample nucleic acid strands useful for diagnosing the presence or absence of disease-associated genetic abnormalities.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassaemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US5589330 [60]	31 Dec 1996	High-throughput screening method for sequence or genetic alterations in nucleic acids using elution and sequencing of complementary oligonucleotides	Anthony P. Shuber	Genzyme Corporation	The present invention pertains to high-throughput screening methods to identify genetic alterations in DNA samples. A multiplicity of DNA samples are immobilized on a solid support and hybridized simultaneously with a mixture of oligonucleotides representing variant sequences. Hybridized oligonucleotides are then eluted from each DNA sample individually and their sequence determined, allowing the identification of disease-causing mutations and polymorphisms in patients' DNA.	The present invention encompasses high-throughput methods for detecting genetic alterations (defined as nucleotide additions, deletions, or substitutions) in a large number of DNA samples. In current clinical practice, different clinical syndromes, for example, thalassaemia, are screened independently of each other.
US5665540 [61] Published also as: US5654148 WO199302134	9 Sep 1997	Multicolor in situ hybridization methods for genetic testing	Roger V. Lebo	The Regents Of The University Of California	This invention relates to novel methods of optimally analyzing commonly obtained prenatal cell samples by in situ hybridization. In addition, this method diagnoses gene deletions and gene duplications using multicolor in situ hybridization. A method is also provided to use multicolor in situ hybridization to identify chromosomal haplotypes co-segregating with disease-related genetic alterations and with normal genes.	Fixation methods for in situ hybridization to amniocytes, chorionic villus cells, fibroblasts, and blastocysts are included for diagnostic and research applications. Using in situ hybridization to uncultured amniocytes and chorionic villus cells provides accurate results quickly from small amniotic fluid volumes or a few villi without expensive, time consuming cell culture.
US5849483 [62] Published also as: CA2195880 DE69531831 EP0777750 WO1996003529	15 Dec 1998	High throughput screening method for sequences or genetic alterations in nucleic acids	Anthony P. Shuber	Ig Laboratories, Inc.	The patent describes a high-throughput method for screening nucleic acid samples to identify target sequences or one or more genetic alterations in target sequences in the nucleic acid samples. Methods of identifying target nucleic acid sequences in patient samples, and of identifying randomly permuted alterations in nucleic acid sequences of interest are also included.	The present invention encompasses a high-throughput method for a rapid and economical screening of nucleic acid samples (generally DNA isolated from a patient) for target sequences of interest. The method is applicable for genomic DNA regions involved in a pathological condition or syndrome as SCA and β -thalassaemia.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US6485901 [63] Published also as: DE69828432 EP1027372 EP1484337 US6649349 US7422852 US7892741 US20020064772 US20070178471 US20090136941 US20110250596 WO1999021881	26 Nov 2002	Methods, kits and compositions pertaining to linear beacons	Brian D. Gildea, James M. Coull, Jens J. Hyldig-Nielsen, Mark J. Flاندaca	Boston Probes, Inc.	This invention is directed to methods, kits and compositions pertaining to Linear Beacons. In the absence of a target sequence, Linear Beacons facilitate efficient energy transfer between the donor and acceptor moieties linked to opposite ends of the probe. Upon hybridization of the probe to a target sequence, there is a measurable change in at least one property of at least one donor or acceptor moiety of the probe which can be used to detect, identify or quantitate the target sequence in a sample.	This invention is related to a method for the detection, identification or quantitation of a target sequence in a sample. Kits and compositions are particularly useful for the rapid, sensitive, reliable and versatile detection of target sequences which are specific for a genetically based disease or for a predisposition to a genetically based disease, such as β -thalassemia and SCA.
US20030186230 [64]	2 Oct 2003	Medium and low density gene chips	Ye Bangce	Ye Bangce	In comparison with current techniques, in this invention a 70×4 DNA probe is fixed on the surface of a DNA chip with the size of a microscope slide, and this probe can detect hereditary anemias, such as α - or β -thalassemia, and HbA abnormalities caused by related gene mutation. The invention has the characteristics of parallel multiple analysis. The completely matched and single-base-non-matched hybridization can be distinguished.	In the existing techniques for diagnosing relevant disease-related gene mutations, there exist the problems of complicated operation, long operating time needed, high cost and difficulty for the realization of automation and mass-sample parallel analysis. The purpose of this invention is to provide a DNA chip for simultaneously testing all hereditary anemia related gene mutations for diagnosis. This DNA chip is appropriate for early diagnosis and prenatal screening.
US20040146883 [65]	29 Jul 2004	Methods for prenatal diagnosis	Giulia Kennedy	Affymetrix, INC.	A prenatal DNA sample is genotyped on microarrays and genetic abnormalities are diagnosed. A method and a kit for prenatal diagnosis are provided, in order to obtain a prenatal nucleic acid sample and to genotype at least 500 – 10000 SNPs in the sample.	The use of WGS (Whole Genome Sampling Assay) genotyping technology, in conjunction with current prenatal diagnosis methods, can be used in prenatal diagnosis.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassaemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US7374765 [66] Published also as: US6849399 US20050090430	20 May 2008	Methods and compositions for diagnosis and treatment of iron misregulation diseases	John N. Feder, Randall C. Schatzman, Zenta Tsuchihashi	Bio-Rad Laboratories, Inc.	Methods and compositions are provided for the diagnosis and treatment of iron misregulation diseases, including HFE polypeptides, agonists, and antagonists, and transferrin receptor agonists and antagonists.	An object of the instant invention is to provide a molecular basis for the relationship of HFE to iron metabolism, and diagnostic and therapeutic agents for the treatment of iron misregulation diseases.
US20090069639 [67] Published also as: EP2202523A1	12 Mar 2009	Diagnosis assisting system, diagnosis assisting information providing device and computer program product	Jo Linssen, Matthias Guhl	Symex Corporation	The approach consists of an analyzing section on a sample collected from a subject, a determining section for presence of a predetermined disease and a notification section. Finally, a diagnosis assisting information will display the obtained information related to the predetermined disease.	The present invention relates to a diagnosis assisting system able to help physicians to analyze and understand examination data in order to correctly diagnose the condition of a patient.
US7888009 [68] Published also as: US6852487 US7083917 US7879579 US7892746 US7892747 US7893233 US7914981 US8288521 US8624016 US8703928 US20140100135	15 Feb 2011	Detection of nucleic acid sequence differences using the ligase detection reaction with addressable arrays	Francis Barany, George Barany, Robert P. Hammer, Maria Kempe, Herman Blok, Monib Zivi	Cornell Research Foundation, Inc.	The ligation phase utilizes a ligation detection reaction between one oligonucleotide probe, which has a target sequence-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target sequence-specific portion and a detectable label. After the ligation phase, the capture phase is carried out by hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. Following completion of the capture phase, a detection phase is carried out.	The diagnostic method includes a ligation phase, a capture phase, and a detection phase for the detection of nucleic acid sequence differences in nucleic acids by one or more single base changes. It has a number of advantages over prior art systems, particularly its ability to carry out multiplex analyses of complex genetic systems. As a result, a large number of nucleotide sequence differences in a sample can be detected at one time.
US8304197 [69] Published also as: CN101076730 US7320894 US7411048 US7649081 US8003338	6 Nov 2012	Diagnostic method for diseases by screening for hepcidin in human or animal tissues, blood or body fluids and therapeutic uses therefor	Hasan Kulaksiz, Cyril E. Geacintov, Alfred Janetzko, Wolfgang Stremmel	Drg International, Inc.	The present invention concerns hepcidin regulation of iron uptake by mammalian cells and the use of hepcidin and/or hepcidin specific antibodies in the diagnosis of diseases involving disturbances of iron metabolism.	The diagnostic detection kits of the present invention can be particularly useful in screening the overall population of either humans or animals and identifying those subjects who have iron diseases.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
US8603773 [70] Published also as: CN102822670 EP2561350 US20100240055 US20140065665 WO201119163	10 Dec 2013	Method and system for analyzing a blood sample	Christophe P. Godefroy, John Steven Riley, Patricio J. Vidal	Beckman Coulter	Methods, systems, and computer program products for the analysis of a blood sample are disclosed. One embodiment is a method of detecting and enumerating hard-to-ghost cells in a blood sample. Another embodiment is a method for analyzing reticulocytes in a blood sample. Methods for using blood count parameters are also provided.	This invention relates in general to the methods and systems for detecting blood pathologies, such as thalassemia, Cooley's disease, and SCA (HbS disease), as well as various clotting and bleeding disorders. Early diagnosis of these disorders is critical so patients who have the disease can receive proper treatment and disease management.
WO2013052557 [71] Published also as: US20140206552	11 Apr 2013	Methods for preimplantation genetic diagnosis by sequencing	Matthew Rabinowitz, Matthew Hill, Bernhard Zimmermann, Johann Baner, Allison Ryan, George Gemelos	Natera Inc, Matthew Rabinowitz, Matthew Hill, Bernhard Zimmermann, Johann Baner, Allison Ryan, George Gemelos	The present invention provides methods for determining the ploidy status of an embryo. In an embodiment, the DNA is selectively amplified at a plurality of polymorphic loci by targeted sequencing.	The present disclosure generally relates to methods for preimplantation genetic diagnosis in the context of <i>in vitro</i> fertilization. This method has a number of potential applications, for example to SNP genotyping and heterozygosity rate determination. The method is based on mini-PCR and/or multiplexed PCR.
WO2014052909 [72]	3 Apr 2014	System for genome analysis and genetic disease diagnosis	Stephen King-smore, Neil Miller, Carol Saunders, Sarah Soden, Emily G. Farrow, Laurel K. Willig	The Children's Mercy Hospital	The method for genome analysis translates the clinical findings in the patient into a comprehensive test order for genes that can be causative of the patient's illness, delimits analysis of variants identified in the patient's genome to those that are 'on target' for the patient's illness, and provides clinical annotation of the likely causative variants for inclusion in a variant warehouse.	The system of the present invention for comprehensive mapping of clinical features to potentially causative genes allows the analysis and interpretation of genetic variants by a factor of 100-fold to 10,000-fold. This greatly decreases the time and effort in interpreting genetic variations that are clinically relevant in WGS.
US8759002 [73] Published also as: EP2384368 US20120094286 WO2010086410	24 Jun 2014	Gene dosage analysis	Andreas R. Huber, Luca Bernasconi, Roberto Herklotz, Saskia Brunner-Agten	Kantonsspital Aarau Ag	The present invention relates to methods of quantitative detection of a genetic polymorphism within two or more closely linked, homologous genes, for example α -globin genes in case of α -thalassemia, in a sample using RT-PCR by subjecting the sample to separate amplification reactions using (a) a pair of forward and reverse primers specific for the head region of each of said two or more closely linked, homologous genes and (b) a pair of forward and reverse primers specific for the tail region of each of said two or more closely linked, homologous genes.	The invention relates to methods for the differential quantitative detection of multiple, closely linked genes, in particular to methods that allow rapid and accurate genotyping of the alleles of the highly homologous α -chain HbA genes, the globin genes $\alpha 1$ and $\alpha 2$.

PCR: Polymerase chain reaction.

Table 2. Partial list of patents and patent applications on novel strategies and specific methods for diagnosis of β -thalassemia (continued).

Patent or patent application	Date	Title	Inventors	Original assignee or co-assignee	Short description (claims)	Validity, significance and biomedical applications
EP1130113 [74] Published also as: CA2400240 EP1255871 US6955901 US20030108913 WO2001061033	5 Sep 2001	Multiplex ligation dependent amplification assay	Johannes Petrus Schouten	Johannes Petrus Schouten	The first nucleic acid probe is complementary to a first part of the target nucleic acid and the second nucleic acid probe is complementary to a second part of the target nucleic acid located essentially adjacent to the first part. The probes further comprise a tag, which is essentially non-complementary to the target nucleic acid, for post-hybridization amplification of the signal.	A method for detecting the presence of at least one single stranded target nucleic acid in a sample.
US8722334 [75] Published also as: CA2694619 CN101855363 EP2183387 US20090053719 US20110183330 WO2009019455	13 May 2014	Analysis for nucleic acids by digital PCR	Yuk Ming Dennis Lo, Rossa Wai Kwun Chiu	The Chinese University of Hong Kong	A method for determining in a sample taken from a pregnant woman the relative amount of a target gene, comprising the steps of: (i) preparing the sample; (ii) treating the sample with a methylation-sensitive restriction enzyme, which cleaves the target gene at a predetermined location only when there is no methylation; (iii) performing amplification reactions; (iv) detecting amplified nucleotide sequence.	The present invention provides a method for analyzing nucleic acids for their lengths and relative abundance in a sample, based on digital amplification of individual template molecules. This invention has many applications, including those in non-invasive prenatal diagnosis, transplantation monitoring, and the detection and monitoring of cancers and virus-associated diseases.

PCR: Polymerase chain reaction.

genetic analysis at all the stages of the patient's life (including non-invasive prenatal diagnosis) is a key issue.

When genetic tests are performed on adult or newborn patients (Figure 1A), only a peripheral blood sample is required in order to isolate DNA to be analyzed. The major issue of genetic analyses involving adult patients is the molecular characterization of the disease, eventually linking diagnosis to prognosis and personalized therapy [5,11]. In this case, usually genetic testing is performed after the onset of the pathology, when clinical symptoms become evident. On the contrary, if newborns are screened for inherited disorders, the possibility for pre-symptomatic identification and early intervention may allow us to prevent or mitigate morbidity and mortality [6]. This approach is particularly important in the case of SCA, when screening for the condition in the neonatal period would enable early diagnosis and therefore, as there are a number of potentially serious complications associated with the condition, is crucial for early life-saving treatments.

When genetic testing is used in prenatal diagnosis (Figure 1B), cells from embryos or fetuses are analyzed in order to identify genetic diseases before birth [7-10]. As a therapeutic treatment is not yet currently available for several pathologies, screening tests should be offered to all pregnant women to assess their risk of having a baby with a birth defect or a genetic disorder [7]. In this respect, it should be underlined that intrauterine therapy can be offered in some cases in which severe fetal abnormalities are prenatally diagnosed, for which natural history anticipates a fatal outcome or the development of severe disability despite optimal postnatal care [11]. Fetal therapy has therefore developed as an alternative to fatalist expectant prenatal management as well as to termination of pregnancy (TOP). Currently, prenatal diagnosis is performed after invasive sampling techniques, such as amniocentesis or chorionic villus sampling, which allow us to collect cells from embryos or fetuses to be analyzed [8]. However, as invasive methods carry a significant risk of miscarriage, non-invasive sampling techniques have been recently developed [9]. They require only a peripheral blood sample from the pregnant woman, in order to isolate fetal cells or cell-free circulating fetal DNA, without any risk for the mother or the fetus. Although we should consider that prenatal diagnosis using fetal cells or cell-free fetal DNA in the maternal circulation has still many technical problems (mainly the very low number of fetal cells and the maternal DNA contamination) that should be solved before its employment in daily use in clinical settings, this technology has been object of great research efforts in recent years [8].

On the other hand, the technological progress in genetic testing and the advent of PGD technologies (Figure 1C)

[12,13], in the nineties, has allowed couples carrier of genetic diseases to select unaffected embryos for transfer before implantation, after an *in vitro* fertilization (IVF) [14,15]. This procedure is particularly suitable for couples who would like to screen their embryos for aneuploidies before implantation or at risk for monogenic diseases but averse to abortion of a possibly affected fetus after prenatal diagnosis. Finally, genetic testing can be performed on pre-fertilized oocytes (Figure 1D), in order to identify the oocyte free of the genetic mutation under analysis to be undergone to programmed fertilization [14,15].

A final comment is related to the emerging field of investigation concerning the so-called genetic modifiers [43]. With respect to this issue, there is considerable evidence that significant factors underlying the individual variability in response to disease, therapy and prognosis lie in a person's genetic make-up and there have been numerous examples of polymorphisms within a given gene altering the functionality of the protein encoded by that gene, and thus leading to a variable physiological response. Furthermore, polymorphisms associated to other 'genetic modifiers' (for example, in the case of β -thalassemia, BCL11A and MYB) have been studied in deep and support the concept of an urgent need of integration of genomics into medical practice, enabling design and building of technology platforms useful for the everyday practice of molecular medicine bringing the basic therapeutic strategies to personalized interventions. It is becoming clear that DNA sequence data aligned with the identification of genes central to the induction, development, progression and outcome of β -thalassemia will be a key point for directing personalized therapy.

Declaration of interest

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