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A new case of autosomal recessive agammaglobulinaemia with impaired pre-B cell differentiation due to a large deletion of the IGH locus

Abstract Males with X-linked agammaglobulinaemia (XLA) due to mutations in the Bruton tyrosine kinase gene constitute the major group of congenital hypogammaglobulinaemia with absence of peripheral B cells. In these cases, blockages between the pro-B and pre-B cell stage in the bone marrow are found. The remaining male and female cases clinically similar to XLA represent a genotypically heterogeneous group of diseases. In these patients, various autosomal recessive disorders have been identified such as mutations affecting *IGHM*, *CD79A*, *IGLL1* genes involved in the composition of the pre-B cell receptor (pre-BCR) or the *BLNK* gene implicated in pre-BCR signal transduction. In this paper, we report on a young female patient characterised by a severe non-XLA agammaglobulinaemia that represents a new case of Igμ defect. We show that the B cell blockage at the pro-B to pre-B cell transition is due to a large homologous deletion in the *IGH* locus encompassing the *IGHM* gene leading to the inability to form a functional pre-BCR. The deletion extends from the beginning of the diversity (D) region to the *IGHG2* gene, with all *JH* segments and *IGHM*, *IGHD*, *IGHG3* and *IGHG1* genes missing. *Conclusion:* alteration in Igμ expression seems to be relatively frequent and could account for most of the reported cases of autosomal recessive agammaglobulinaemia.

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Introduction

Recurrent infections, mostly respiratory, with pyogenic bacteria are the predominant manifestations of children suffering antibody deficiencies. In some of these patients, early B cell development in the bone marrow is arrested and hypogammaglobulinaemia results from the absence of peripheral B cells. In these cases, chronic diarrhoea could also be observed and constitutes a serious clinical problem. This occurs in X-linked agammaglobulinaemia (XLA), also known as Bruton disease, characterised by defects in the Bruton tyrosine kinase gene (BTK) [13,14] that encodes the cytoplasmic tyrosine kinase btk, involved in signal transduction. In these cases, B cell development is blocked in the bone marrow at the pre-B cell stage, resulting in the accumulation of CD34+ CD19+ pro-B cells and in the presence of variable numbers of CD34-CD19+ pre-B cells. Females with a phenotype indistinguishable from XLA have also been described and Conley et al. [2] have estimated that these immunodeficiencies represent 10% of patients with congenital hypogammaglobulinaemia. The defects behind such autosomal recessive disorders have been recently identified and shown to affect predominantly the pre-B cell receptor (pre-BCR), which is an absolute prerequisite for pro-B to pre-B cell transition and to allow further B cell differentiation [3,12]. This receptor, expressed on pre-B cells is composed of the Igμ, the ΨL chain (made of λ-like and VpreB) and the Igα/Igβ transducing complex [5]. For example, mutations affecting *IGHM* [12,15], *CD79A* [10], *IGLL1* [9] or

BLNK that encodes a B cell linker protein essential for Igµ signal transduction [11], have been reported.

In this paper we describe a new case of autosomal recessive agammaglobulinaemia due to a large deletion of the immunoglobulin heavy chain locus (IGH), including the *IGHM* gene, that results in the absence of a functional pre-BCR.

Case report

The patient, a 35-month-old girl, was the second child of healthy parents born in the same small village, near Braga, Portugal, who were thought to be non-consanguineous (see below). She had a healthy 6-year older brother. Apart from symmetrical intrauterine growth retardation due to uteroplacental insufficiency, no other significant abnormalities related to family history, pregnancy and delivery were detected. An echocardiogram confirmed a congenital cardiopathy, secundum ASD (atrium secundum, intra-auricular communication) which resolved spontaneously without surgery. At 9 months of age she was referred to the Paediatric Gastroenterology Department because of failure to thrive and chronic diarrhoea during the previous month. Her weight was 5 640 g (P < 3), her height 60 cm (P < 3) and her head circumference 40.8 cm (P < 5). Physical examination showed that she was not dehydrated, she had no detectable tonsils and no palpable adenomegaly and revealed that she had elongated fingers (data not shown). According to the Kanawati-McLaren index (0.31), she had no clinical signs of malnutrition.

A routine blood examination showed leucocyte and platelet counts of 27500/mm^3 and 345000/mm^3, respectively, and haemoglobin at 13.2 g/dl. Total protein was 5.2 g/dl and albumin 3.7 g/dl. Blood, urine and stool cultures were sterile. TORCH serology and sweat tests were negative. Search in stools for virus, parasites, reducing sugar and fat was negative. Alpha-1 anti-trypsin in stools was < 1.7 mg/g dry weight (normal < 5.0 mg/g). Histology of a duodenal biopsy showed no histological changes but immunocytochemistry revealed a complete absence of B cells and normal numbers of T cells. The patient responds to BCG and oral poliomyelitis vaccines without any complication.

Her peripheral blood immunological data, determined at 10 months of age, are presented in Table 1. A clear agammaglobulinaemia was evident with a serum IgG level of 5 mg/dl and undetectable IgM and IgA. Lymphocyte phenotyping showed the absence of circulating B cells (CD19 or CD20 positive cells), normal numbers of CD4 T lymphocytes and increased numbers and proportions of NK and CD8 T cells, this latter population showing increased expression of HLA-DR, CD45RO and CD95 activation markers (data not shown).

Table 1. Immunological data

	Patient (aged 10 months)	Age-matched controls
Lymphocyte count (10^3 cells/µl)	1.1	3–8
Serum immunoglobulins (mg/dl)		
IgM	< 0.1	25–182
IgG	5	441–880
IgA	< 0.1	11–117
Blood B lymphocytes (%)		
CD19	< 0.01	19–31
CD20	< 0.01	19–31
Blood T lymphocytes (%)		
CD3	84	55–79
CD4+/CD3+	46	35–59
CD8+/CD3+	38	16–34
Blood NK lymphocytes (%)		
CD3-/CD16+CD56+	16	4–11

Since the age of 10 months, she has been treated with intravenous replacement immunoglobulin therapy (400 mg/kg every 3 weeks, with pre-administration levels of 300–500 mg/dl). During the 25 months follow-up, two episodes of gastroenteritis, a viral rhinitis and an ear infection were diagnosed, which, however, did not require anti-microbial therapy.

The parents gave informed consent for this investigation.

Materials and methods

Immunoglobulin measurement and cell phenotyping

Serum immunoglobulin IgG, IgA, and IgM were determined by rate nephelometry. Whole peripheral blood and bone marrow mononuclear cells isolated by Ficoll-Hypaque gradient were used for phenotyping. Cell surface antigens were stained with conjugated anti-human monoclonal antibodies and were analysed by flow cytometry. Bone marrow mononuclear cells were stained with FITC-conjugated monoclonal antibodies against CD10, CD20, CD34, CD3, CD4 or phycoerythrin-conjugated monoclonal antibodies against CD19, CD8, CD16 and CD56, from Immunotech (France). Phycoerythrin-conjugated anti-Igδ and APC-conjugated anti-CD19 were from Pharmingen (France). Biotin-conjugated anti-human Igµ chain from Southern Biotechnology (USA) was revealed with PerCP-conjugated Streptavidin (Becton and Dickinson, USA). Peripheral blood B cells were stained with FITC-conjugated monoclonal antibodies against CD19, CD20, CD4, CD8, phycoerythrin-conjugated monoclonal antibodies against CD8, CD16, CD56, PE/Cy5-conjugated monoclonal antibodies against CD3, from Immunotech (France) and from Dako, SA (Denmark), and were haemolysed and fixed (T–prep, Beckman-Coulter) before flow cytometric analysis.

RNA and reverse transcriptase polymerase chain reaction

Total RNA was extracted from total bone marrow cells using TRIzol Reagent (Gibco BRL) as described by Chomczynski and Sacchi [1]. RNA (2 µg) was reverse transcribed using the reverse transcriptase Superscript II (Gibco BRL), 1 µg of random hexamer (dN6), 1 mM dNTPs and the supplied buffer. For reverse transcriptase-PCR, 1 µl of cDNA was amplified for 30 cycles of 30 s at 94°C, 1 min at the appropriate temperature and 2 min at 72°C with a final 10 min extension at 72°C, using Taq DNA polymerase (BRL) and the primers already described [12].

DNA and DNA-polymerase chain reaction

Genomic DNA was extracted from peripheral blood mononuclear cells with proteinase K, sodium dodecyl sulphate and phenol-chloroform extractions. PCR was performed, as above, using 100 ng of DNA. For semi quantitative analysis, PCRs were carried out during the exponential phase of the DNA amplification. In that case, the exponential phase of each PCR was determined by titration of cycle numbers and by quantities of total DNA. PCR products were analysed on 2% agarose gels and revealed by ethidium bromide (BET) staining. Quantification was performed using the MacBas software.

Results

Phenotypic analysis of bone marrow cells

Peripheral blood phenotype of the patient revealed a pure B cell defect since a profound agammaglobulinaemia with a total absence of peripheral B cells was observed and since the levels and phenotype of T and

NK cells did not indicate a T-B-combined immunodeficiency. Thus, we analysed bone marrow B cell subpopulations. A bone marrow sample was obtained when the patient (from now referred as the Igμ$^\Delta$ patient, see below) was 24 months old. Total CD19+ patient's B cells were diminished more than three-fold (13% versus 43% in age matched control) and the population of large B cells, mainly corresponding to proliferating pre-B cells, was nearly absent (Fig. 1A). Gated CD19+ cells were completely negative for surface Igμ expression (Fig. 1B) and for Igδ and CD40 expression (data not shown), indicating a total absence of immature and mature B cells in the patient's bone marrow. In contrast, CD34+ precursors and CD34+CD19+ pro-B cells were within normal limits as compared to an age-matched control (Fig. 1C). Altogether these data indicate that the patient suffered from an early bone marrow B cell blockage, between pro-B and pre-B cell stages.

Molecular analysis

RT-PCR analysis was performed on total mononuclear bone marrow cells for transcripts expressed during the pro-B to pre-B cell transition, including λ-like, VpreB, Igα, Igβ, and Blnk. The resulting PCR products were directly sequenced and were normal (data not shown). In contrast, no transcripts were detected for IgCμ specific amplification. This result was confirmed at the DNA level since no amplification could be detected using the μCH1 and μCH2 oligonucleotides (Table 2, Fig. 2) for the patient's DNA. These data indicate that a deletion within the IGH locus, carried by both alleles, was responsible for the patient's phenotype.

To analyse the extent of the DNA deletion, we performed a detailed analysis of the IGH locus by PCR. By using JH5-JH6 and δCH2-δCH3 primers, we showed that the JH cluster and the IGHD gene respectively were

completely missing. Moreover, we mapped the 5' end of the deletion between the IGHD2–2 and the IGHD3–10 genes within the IGHD locus, since we could amplify the IGHD2–2 and not the IGHD3–10 gene (Fig. 2). Using γCH2 and γCH3 oligonucleotides that are common to different IGHG gene sub-classes, we demonstrated that some IGHG genes were present in the patient's genome.
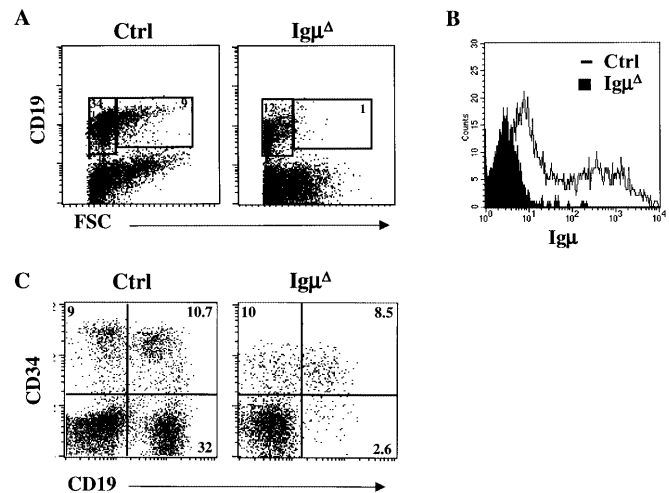
Fig. 1. FACS analysis of bone marrow B cell sub-populations for the Igμ$^\Delta$ patient and an age-matched control (*Ctrl*). **A** Bone marrow mononuclear cells from Igμ$^\Delta$ and an age-matched control were stained with anti-CD19 monoclonal antibody and analysed by flow cytometry. Fluorescence intensity of stained cells is presented as a function of cell size (forward scatter). **B** Bone marrow mononuclear cells were stained with anti-CD19 and anti-Igμ monoclonal antibodies and CD19+ cells, gated and analysed for Igμ chain expression. *Solid* and *empty* curves represent Igμ$^\Delta$ patient and age-matched control, respectively. **C** Bone marrow mononuclear cells were stained with anti-CD34 and anti-CD19 monoclonal antibodies. Dot plots show precursor (CD34+), pro-B (CD34+CD19+) and pre-B+immature+mature (CD34-CD19+) bone marrow B cell sub-populations

Table 2. Oligonucleotide primers used for DNA-PCR amplification

Locus	Form	Primers used (5' to 3')	Annealing temperature (°C)	Product size (bp)
D 2-2	Sense	GATATTGTAGTAGTACCAGCTGC	66	422
	Antisense	GTAGGATAGCGGCTGCTTCTC	66	
D3-10	Sense	GTATTACTATGGTTCGGGGAG	62	564
	Antisense	GAAATGACCCAGGAGAGGCTC	66	
JH5-JH6	Sense	GCCGTCTGCTTGCAGTTGGAC	68	593
	Antisense	CTACAGACACCGCTCCTGAGAC	70	
μCH1-μCH2	Sense	AGGGAGTGCATCCGCCCCAAC	68	647
	Antisense	CTTTGATGGTCAGTGTGCTGGTC	70	
δCH2-δCH3	Sense	GCAGTGCAGGACCTGTGGCTC	70	577
	Antisense	GCCAGACACCTCACACAGGAGC	72	
γCH2-γCH3	Sense	GGAGGTGCATAATGCCAAGACAAAG	74	360
	Antisense	TTGACCAGGCAGGTCAGGCTGAC	74	
Sγ1	Sense	GCAGAGCATCACAACGTCAGC	66	261
	Antisense	GAGCTAGTCCTGCTCACCTTC	66	
γ2H-γ2CH2	Sense	GCGCAAATGTTGTGTCGAGT	60	175
	Antisense	CGGTCCTGCCACAGGTGG	62	
VpreB	Sense	CTGAGATATTTCTCACAATCAGAC	66	284
	Antisense	TCTTCCATGACAGTCTCTCCAAG	68	
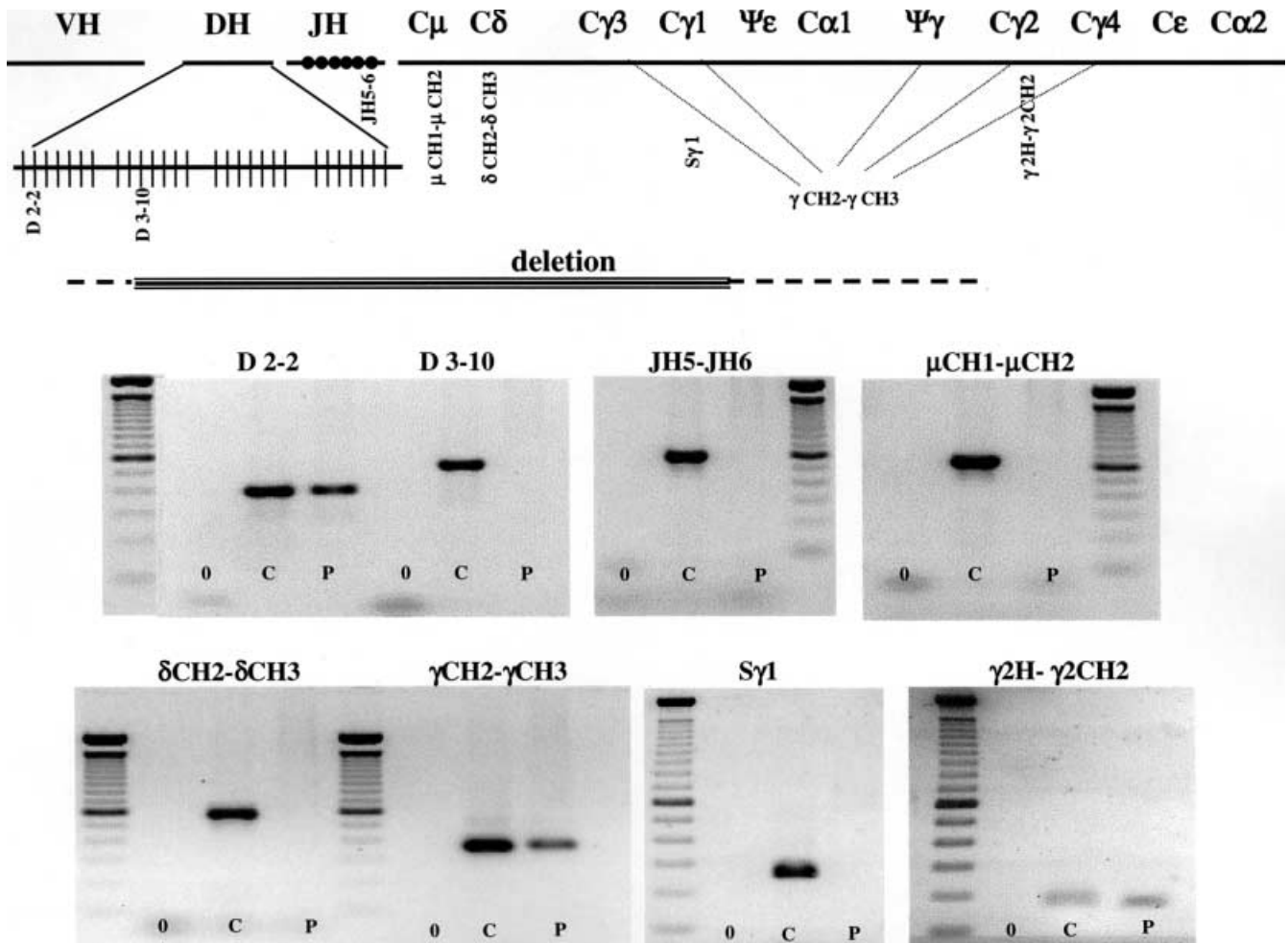
Fig. 2. Molecular analysis of the patient's gene defect. Schematic representation (*top*) of the human *IGH* locus and localisation of the primers used for DNA amplification. Extent of the Igμ$^\Delta$ patient DNA deletion is shown. PCR amplification (*bottom*) of the Igμ$^\Delta$ patient DNA using the indicated oligonucleotides (see Table 2). 0, C and P lanes correspond to PCR without DNA, control DNA and Igμ$^\Delta$ patient DNA, respectively. Amplified DNA products were separated on 2% agarose gels and revealed by BET staining

Using specific primers in the *IGHG1* switch region (Sγ1) or in the hinge region of the *IGHG2* gene (γ2H and γ2CH2), we finally demonstrated that the 3' end of the DNA deletion was located between the *IGHG1* and the *IGHG2* genes (Fig. 2). The size of the DNA deletion was estimated to be between 165 to 265 kb [6].

Family studies

The PCR results obtained for the Igμ$^\Delta$ patient suggested the presence of a large homologous DNA deletion of familial origin. A semi-quantitative PCR was performed using VpreB and Igμ specific primers on the DNA extracted from peripheral blood mononuclear cells of the father, mother, brother and a healthy donor (Fig. 3A). Since *VPREB* is a unique gene in the human genome [4]

it was used to quantify Igμ PCR products. The results clearly demonstrated that the DNA from both the father and the mother contained only one copy of the functional *IGHM* gene (Fig. 3B). In contrast, DNA of the brother as well as control DNA contained two copies of the *IGHM* allele (Fig. 3B). The patient's brother was therefore homozygous for the functional *IGHM* allele.

Discussion

Approximately 85% of patients with congenital hypogammaglobulinaemia and absent peripheral B cells are males with XLA due to mutations in the *BTK* gene. The remaining 15% of cases, clinically identical to XLA, are still a heterogeneous group. Some mutations have been already identified, especially in components of the pre-BCR, such as *IGHM* [12,15], *IGLL1* [9], *CD79A* [10], or *BLNK* [11] that result in impaired pre-B cell differentiation. However, alteration in Igμ expression seems to be relatively frequent. For example, Yel et al. [15] described three different mutations in the *IGH* locus. For one consanguineous family, the affected patients had a DNA deletion covering 75–100 kb that encompassed the diversity genes (DH), the JH cluster and the *IGHM* gene.

Fig. 3. Molecular analysis of family's patient DNA at the *IGH* locus. **A** PCR was performed on DNA extracts from healthy donor's peripheral blood mononuclear cells (*C*), father (*F*), mother (*M*) brother (*B*) and patient (*P*) using μCH1-μCH2 and VpreB specific primers (see Table 2). 0 corresponds to PCR without DNA. Amplified DNA products were separated on 2% agarose gels and revealed by BET staining. **B** For each DNA, Igμ specific amplification is quantified according the VpreB amplification
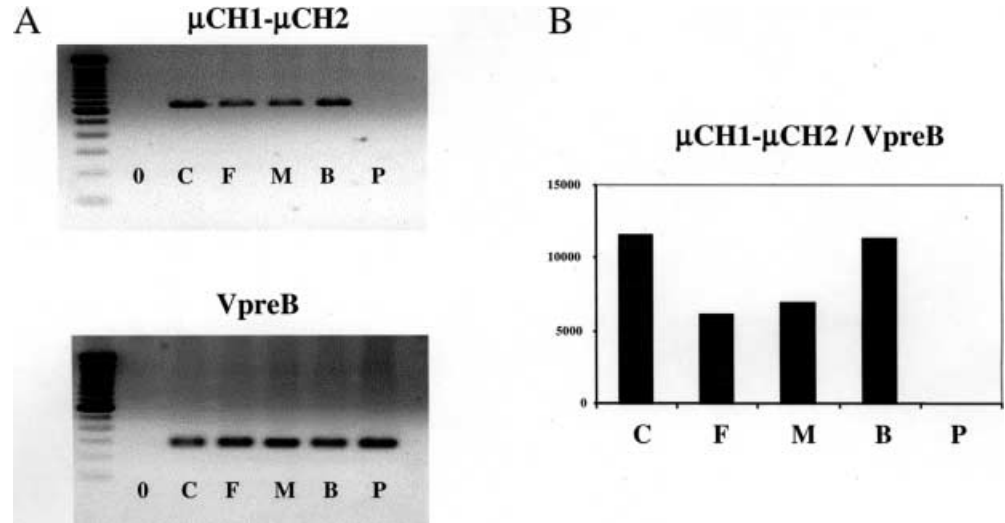
For another consanguineous family, the alteration corresponded to a homozygous single G to A base pair substitution at the –1 position of the alternative splice site used to produce the membrane form of the Igμ transcript. This mutation would inhibit the production of the membrane Igμ chain. It was also reported that a boy, who did not suffer from a *BTK* alteration, had a heterozygous alteration of the *IGHM* gene. In this case, a single base pair substitution affecting an invariant cysteine residue in the CH4 domain of the *IGHM* chain for one allele, coupled to a large deletion of the *IGH* locus on the other allele, was detected. We have also reported the case of a girl, referred to as the Igμ$^{-/-}$ patient [7,12], in whom we identified a cytosine insertion at the beginning of the CH1 exon of the *IGHM* gene, resulting in a stop codon at position 48, and in the absence of Igμ chain expression. The patient reported in this paper, designated as the Igμ$^{\Delta}$ case [8], with a large 165 to 265 kb homologous deletion of the *IGH* locus encompassing the *IGHM* gene, represents a new case of Igμ defect. Altogether we can conclude that Igμ alterations account for most of the reported cases of autosomal recessive agammaglobulinemia. These observations also emphasise the requirement of signalling via a functional pre-BCR to ensure normal early B cell differentiation. Moreover, the absence of Igμ chains in Igμ$^{-/-}$ and Igμ$^{\Delta}$ patients, has provided an opportunity to elucidate the role of Igμ in selecting the antibody repertoire in humans [8].

Along with a better understanding of B cell biology and its developmental defects, identification of such genetic alterations may open the way for new clinical treatments, including somatic gene therapy. Moreover it provides a basis for a more accurate diagnosis, comprising the identification of carrier status, prenatal testing and counselling.

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