CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

1. X-linked agammaglobulinemia (XLA)

X-linked agammaglobulinemia is prototypic inherited primary humoral immunodeficiency characterized by a profound hypogammaglobulinemia, an early onset of bacterial infections, a decrease in the number of B-lymphocytes, and an almost complete lack of plasma cells (REF). It is associated with a failure of Ig heavy chain rearrangement.[2] The immunoglobulin levels in affected individuals are very low. The associated infections, encapsulated bacteria and enterovirus, are often life threatening and therefore, early diagnosis is critical for the management of XLA. [3,4]

The study of XLA has profoundly contributed to the understanding of the immunological basis of the humoral immunity. *BTK* is expressed throughout the development in B cells except the plasma cells [2]. In XLA, the production of *BTK* is blocked at the transitional stage from pro-B to pre-B cells resulting in B cell apoptosis[7] (see Fig 2). Moreover, as recently shown in xid mice, the first manifestation of BTK deficiency occured in the transition from pro-B to pre-B stage. Thus, the initial effects of xid in mice and XLA in man are at the same stage of B cell development [8]. Various types of XLA causing mutations have been identified in all BTK domains.

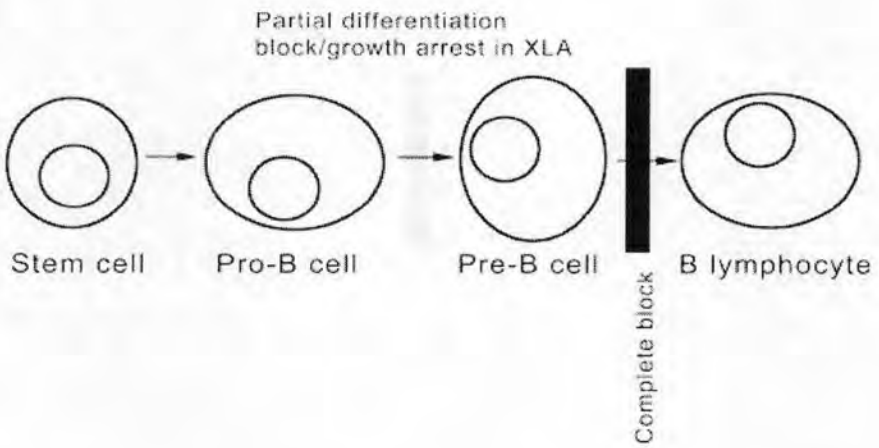

Figure 2: The schematic illustration of B-cell development. BTK is expressed throughout B cell development, but down regulated in plasma cells. Upon *BTK* mutation, there is a block in the development at the transitional stage from pro- to pre- B cells leading to XLA in man and xid in mice.

2. Clinical classification

Patients are unusually prone to bacterial infection but not to viral infection. A clinical picture resembling rheumatoid arthritis develops in many. Before antibiotics, death occurred in the first decade. In the more usual X-linked form of the disease, plasma cells are lacking. Because of passive, transplacental acquisition of maternal IgG, newborns have normal levels of serum IgG and do not have problems until the IgG is catabolized. Because newborns cannot produce their own immunoglobulins, increased susceptibility to infections develops in infants older than 6 months. When the maternal immunoglobulin supply has been depleted, a period of recurrent infections begins in babies with agammaglobulinemia.

2.1 Bacterial infections

As in all forms of immunodeficiency, bacterial infections of the respiratory tract are common. Individuals with X-linked agammaglobulinemia have recurrent ear, throat, sinus and pulmonary infections, often involving "encapsulated" bacteria. Invading polysaccharide-encapsulated bacteria strain the immune system, increasing the need for antibodies. There is also an increased risk of severe bacterial infections, such as sepsis (bloodstream infection), meningitis, osteomyelitis or gastrointestinal infections.

2.2 Particular complications

Although patients with agammaglobulinemia have recurrent bacterial infections, they generally are able to handle viral infections, they are susceptible to certain viruses that replicate in the gastrointestinal tract and then spread to the CNS. This indicates the importance of antibody production in limiting the spread of infections by enteroviruses such as poliovirus, echovirus, and coxsackievirus. [5]

Encephalitis

There is no increased risk of developing fungus or viral infections. One notable exception is the elevated risk for boys and men with X-linked agammaglobulinemia to develop encephalitis. Encephalitis usually results in slowly progressive brain damage, and the condition is fatal. It is not always possible to detect the cause of the infection, but it may be caused by a member of the enterovirus family.

Enteroviruses include the polio virus and certain viruses causing diarrhoea. It should be noted that the live attenuated polio virus found in oral polio vaccine may actually transmit the disease it was designed to prevent in agammaglobulinemia patients. This type of vaccine is not available in Sweden, but is commonly used in other parts of the world.

Mycoplasma infections

People with immunodeficiency may be affected by pneumonia caused by mycoplasma bacteria. Other types of infection caused by species of mycoplasma also present, although most of these bacteria are normally not harmful to humans. Many boys develop joint problems reminiscent of juvenile idiopathic arthritis, and sometimes bacteria are isolated in the synovial fluid or the joint capsule. Mycoplasma bacteria may also cause kidney or urinary tract infections.

Chronic Hemophilus influenzae

Even if immunoglobulin treatment is effective, a man or boy with X-linked agammaglobulinemia is particularly susceptible to respiratory tract infections caused by the bacteria Hemophilus influenzae. Common infections include bronchitis, sinusitis, and conjunctivitis. Recurrent infections may have a long-term deleterious effect on a number of organs, especially the lungs. The prognosis varies and will depend on how early the diagnosis is made and if adequate immunoglobulin therapy is provided. It is also important that infections are properly treated.

3. Incidence and inheritance

X-linked agammaglobulinemia occurs worldwide. The disease afflicts about 1/100,000-20,000 males.

As a rule, X-linked recessive genetic disorders are only found in men, but they are passed down via healthy woman carriers of the mutation (Fig. 3). Sons of female carriers run a 50 per cent risk of inheriting the disorder, and daughters run the same risk of becoming carriers. A man with an X-linked recessive genetic trait cannot pass it down to his sons, but all his daughters will be carriers (Fig. 4).

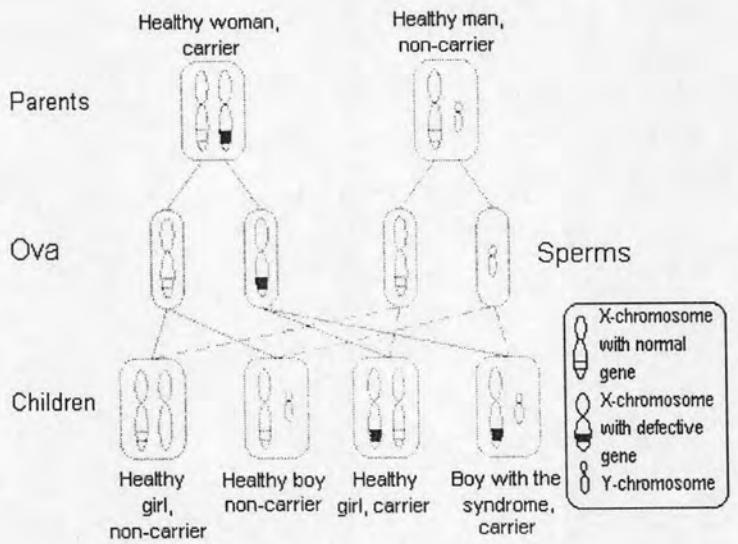

Figure 3: X-linked recessive genetic trait from a healthy woman who is a carrier. (Pictures from SocialStyrelsen)

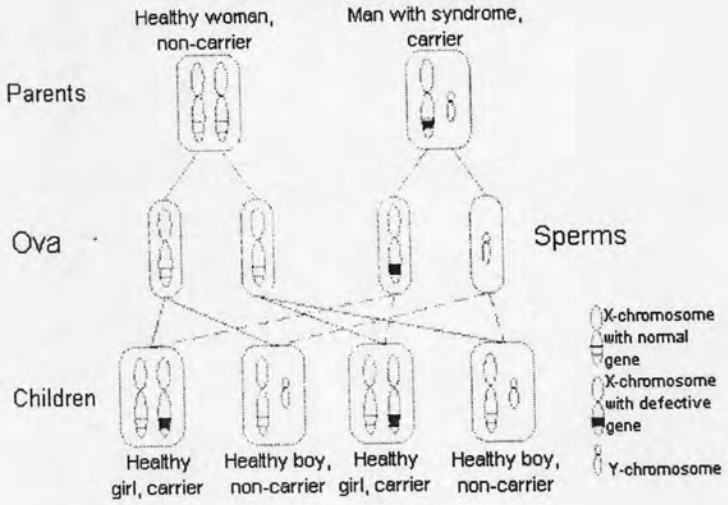

Figure 4: X-linked recessive genetic trait from a man with the syndrome who is a carrier.

X-linked agammaglobulinemia may also be caused by a new mutation, meaning that the defective gene has not been inherited and is not present in the affected individual's family. The risk that the parents of an affected child will have another child with the disease is therefore minimal. However, the new mutation is hereditary and there is a risk that a person with a new mutation will pass it down to his or her children.

In X-linked recessive genetic disorders, the new mutation may have occurred in the affected boy's mother. As the mother has two X chromosomes (one normal), she remains asymptomatic. However, if she has sons, they may inherit the mutation.

4. Etiology and molecular genetics of XLA

Bruton agammaglobulinemia tyrosine kinase structure

Bruton agammaglobulinemia tyrosine kinase (BTK) was identified as the molecular defect in XLA by two different approaches, using positional cloning [4] and in a search for novel protein kinases expressed in B lymphocytes[6]. The gene was mapped to the Xq21.3-22 region in the mid-portion of the long arm of the X-chromosome. The human gene encompasses 37.5 kb and is organized into 19 exons, including a 5' untranslated region (exon 1). BTK is a 659 amino acid long protein in both man and mouse. BTK belongs to the Tec family of related cytoplasmic protein tyrosine kinases Excluding Txk, the family members all consist of five distinct structural domains, from the N-terminus: pleckstrin homology (PH), Techomology (TH), Srchomology3 (SH3), SH2, and the catalytic kinase domain (Fig. 5).

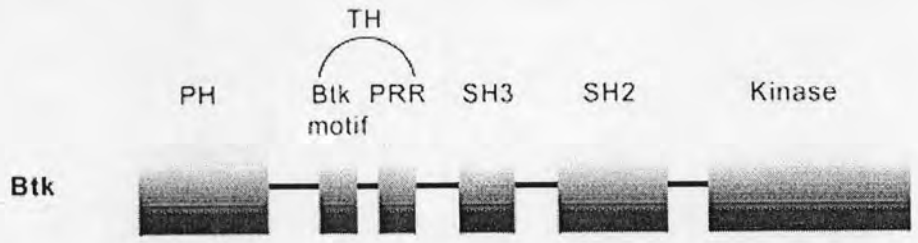

Figure 5: Domain organisation of Bruton tyrosine kinase [23].

The term signal transduction implies the conversion of an input signal received at the extracellular face of the plasma membrane into an intracellular signal that, ultimately, leads to alteration of gene expression. The correct transmission of the signal is vital for both unicellular and multicellular organisms; the stimulation of cells from outside, due to signaling molecules, can trigger a cascade of events leading to diverse cellular responses such as, differentiation, growth and movement[9]. Various types of proteins, including transmembrane receptors, G-protein subunits, adaptor proteins, kinases and phosphatases, are involved in signaling.

The plasma membrane plays an important role as a meeting point for signaling proteins. The association of signaling proteins with the membrane can be direct or indirect. The binding of a cytoplasmic protein to the membrane could be achieved, for example, by modification of the proteins by lipid. Furthermore, with specific protein-protein interactions, signals can also be transduced through the membrane via an indirect mechanism. These processes are regulated through covalent protein modification such as phosphorylation, one of the hallmarks of intracellular signaling[10].

BTK protein is a non-receptor PTK known to be involved in B-cell survival, cell cycle progression and proliferation in response to B cell receptor (BCR) stimulation [11]. The phosphorylation of a tyrosine residue in the activation loop of BTK is essential for its participation in BCR signaling [12].

5. Diagnosis

5.1 All circulating Ig levels (IgG, IgA, IgM, IgE) are low. The patient's specific levels are compared to those in age-appropriate controls.

- Serum IgG levels lower than 100 mg/dL should arouse concern. In some patients with XLA, IgG levels may be as high as 200-300 mg/dL. This does not necessarily exclude a diagnosis of XLA.

- Patients are also unable to make specific antibody responses. Their antibody levels are reduced to common childhood vaccines such as those for diphtheria, pertussis, varicella, hepatitis B, and *H influenzae*.

- In young infants (<6 mo), because the serum IgG level is contaminated from the presence of a maternal antibody, the physician cannot rely on Ig level determinations. Where on the curve (ie, decreasing maternal levels versus increasing infant's level) the value represents is uncertain. Patients' families also have anxiety about a diagnosis of possible immunodeficiency. However, obtaining specific serum diphtheria and tetanus antibody levels before a diphtheria, pertussis, and tetanus vaccine and another set of values 3-4 weeks later is helpful. If specific diphtheria and tetanus levels are increased, the infant is able to produce antigen-specific antibody, making agammaglobulinemia (or any other B-cell deficiency) unlikely.

- Functional IgM production can be measured by checking for isohemagglutinin titers.

- Note that pre–B cells can produce IgM in detectable quantities and that autoantibodies particularly directed against hematopoietic cells (typical antirhesus [anti-Rh] in autoimmune hemolytic anemia, antineutrophil antibodies) are also made.

5.2 Because B-cell maturation is arrested, patients lack mature B lymphocytes in their peripheral blood or tissue. Performing flow cytometry to analyze B- and T-cell markers is necessary.

- This can be assessed by staining for B-lymphocyte–specific surface cell markers by flow cytometry. Most laboratories should be able to perform this test because similar technology examines the T-lymphocyte markers of CD4 and CD8 used in assessing HIV infection. However, laboratory personnel must be informed that B-lymphocyte–specific monoclonal antibodies (CD19 and/or CD20) are needed.

- Reduced numbers of peripheral blood B lymphocytes suggest the diagnosis, no matter what the age of the patient.

5.3 Mutational analysis must be performed to confirm the specific type of agammaglobulinemia.

5.4 In addition, plasma cells and B lymphocytes in lymphoid follicles and in germinal centers of lymph nodes may be lacking. Because intestinal biopsy may be obtained to evaluate patients with chronic diarrhea, examination for hypoplastic Peyer patches in the lamina propria of intestinal mucosa may be suggestive of agammaglobulinemia.

5.5 Patients with growth hormone deficiency have a deficient growth hormone response to insulin, arginine, or levodopa (L-dopa). Plasma somatomedin levels are also reduced.

6. Treatment and intervention

Because a patient with agammaglobulinemia is unable to produce specific antibodies, the primary medical treatment is to replace Ig. Aggressive treatment with antibiotics for bacterial infections may prevent long-term complications. Live viral vaccines are contraindicated in these patients and their families because they may cause vaccine-related infections.

6.1 The intravenous delivery of Ig (IVIG) results in improved clinical status with a decrease in serious infections, such as pneumonia, meningitis, and gastrointestinal infection. This also appears to be the case for hypogammaglobulinemia secondary to malignancy.

Effects and side effects

The first immunoglobulin treatments are sometimes accompanied by chills and fever. If the patient has an infection, these reactions may also present later. This is a normal inflammatory reaction induced by new antibodies beginning to combat the infection, and hardly ever a matter of hypersensitivity. Severe allergic reactions to immunoglobulin are very rare.

6.2 In patients with chronic upper or lower respiratory tract infections and subsequent structural changes, strategic long-term broad-spectrum antibiotics may be needed, in addition to chest physiotherapy and sinus surgery.

- Specific antibiotic choices must cover the usual polysaccharide-encapsulated organisms. Higher doses and longer courses are common.
- Some patients develop chronic sinusitis despite regular IVIG replacement therapy every 3 weeks. These patients are challenging to treat because antibiotics, *N*-acetylcysteine, and topical intranasal corticosteroid therapies fail to clear pathogens and do not decrease sinus inflammation.

7. Splice site mutation

A splice site mutation is a genetic mutation that inserts or deletes a number of nucleotides in the specific site at which splicing of an intron takes place during the processing of precursor messenger RNA into mature messenger RNA. The abolishment of the splicing site results in one or more introns remaining in mature mRNA and may lead to the production of aberrant proteins. Several genetic diseases may be the result of splice site mutations.

In XLA, several different mutations have been observed in the *BTK* gene (Fig. 6). The total number of patients with reported mutations are 620.

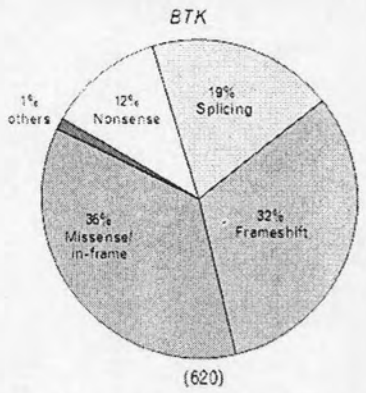

Figure 6: The percentage of each *BTK* mutation is presented for X-linked agammaglobulinemia [13]

Splicing mutations can be grouped into at least five types (Fig. 7). The underlying common denomination being that a critical sequence of nucleotides is disrupted by the mutation so that its chemical entropy is no longer the optimal site for splicing of pre-mRNA. This creates aberrant or cryptic splice sites that translate to aberrant protein.

7.1 Type I refers to the classical splicing mutation that causes the deletion of an entire exon during pre-mRNA splicing.
7.2 Type II splicing mutations occur in the mid-intron and result in the insertion of a pseudoexon at the cDNA level. Blocking antisense morpholino oligonucleotides have been used to restore normal splicing.
7.3 Type III mutations lie within the coding region, resulting in the partial deletion of an exon. An antisense morpholino oligonucleotide has been used to block the mutation generated 5' splicing site.
7.4 Type IV mutations lie within the intron and lead to partial deletion of an exon.
7.5 Type V mutations disturb the lariat branch point and result in deletion of an exon.

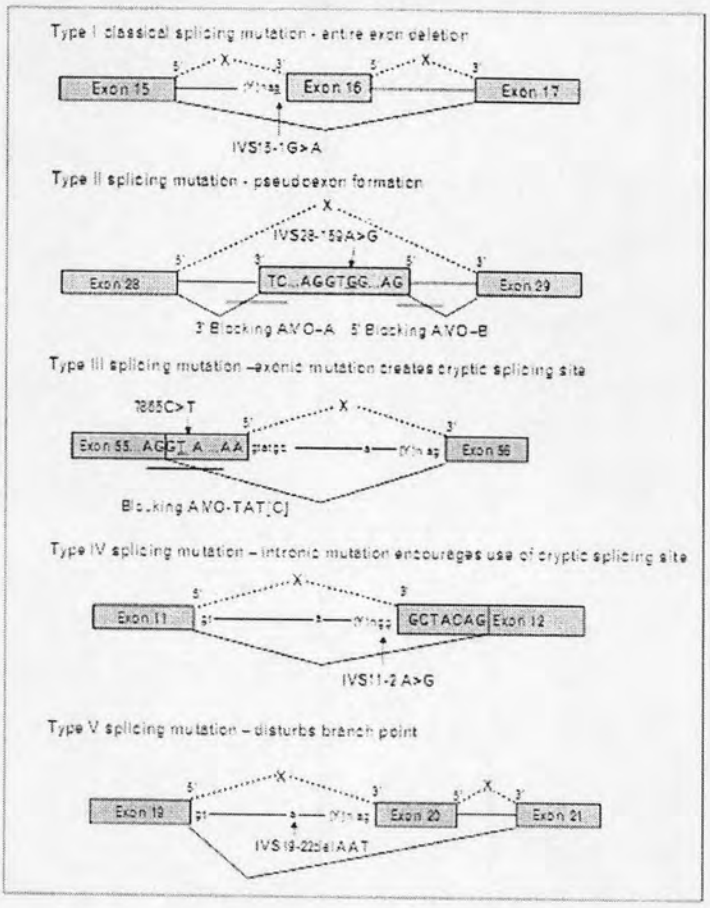

Figure 7: Types of splicing mutations and correction with AMOs [13]

8. Antisense Morpholino Oligonucleotides (AMOs)

Antisense Morpholino Oligonucleotides (AMOs) are molecules used to modify gene expression. Morpholino oligonucleotides (oligos) are an antisense technology used to block access of other molecules to specific sequences within nucleic acid. Morpholinos block small (~25 base) regions of the base-pairing surfaces of ribonucleic acid (RNA). AMOs specifically bind to its selected target site to block access of cell components to that target site. This property can be exploited to block translation, block splicing, block miRNAs or their targets, and block ribozyme activity.

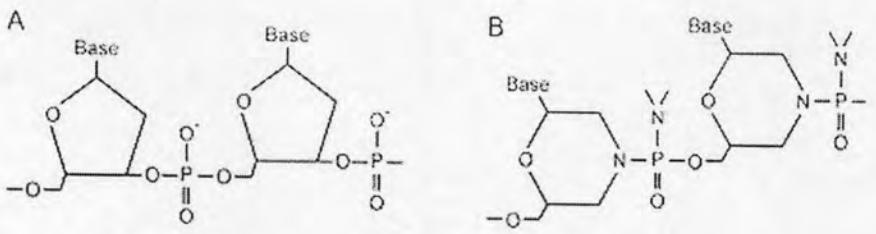

Figure 8: Molecular structure of DNA and AMOs. (A) Conventional DNA oligonucleotide. (B) Morpholino oligonucleotide. Note the six-membered morpholino ring in B and the non-ionic phosphorodiamidate link between the two rings

Translation Blocking: By sterically blocking the translation initiation complex, Morpholinos can knock down expression of many target sequences completely enough that after waiting for existing protein to degrade, the target protein band disappears from Western blots. Unlike many antisense types (e.g. siRNA, phosphorothioates), Morpholinos generally do not cause degradation of their RNA targets; instead, they block the biological activity of the target RNA until that RNA is degraded naturally, which releases the Morpholino. This means that RT-PCR is not suitable for assaying translation blocking by Morpholinos.

Splice Blocking: By blocking sites involved in splicing pre-mRNA, Morpholinos can be used to modify and control normal splicing events. This activity can be conveniently assayed by RT-PCR, with successful splice-modification appearing as band shifts of RT-PCR products on electrophoretic gels.

Currently AMOs have also been used to successfully modulate RNA splicing for many genetic diseases (Table1).

Disease	*Gene*	SSO *modification*	*Study*	*References*[a]
Duchenne muscular dystrophy (DMD)	DMD	2'OMePS	Local and systemic dystrophin induction in mdx mice	(Mann et al., 2001; Lu et al., 2003, 2005)
			A Clinical trial in DMD patients by intramuscular injections	(van Deutekom et al., 2007)
		PMO	Functional level of dystrophin induction in body-wide skeletal muscles in mdx mice after systemic delivery of PMO	(Alter et al., 2006)
			A dose-escalating clinical trial in DMD patients by a single intramuscular injection.	Imperial College of London, UK (in progress)
		PPMO	Effective dystrophin restoration in body-wide muscles including cardiac muscle of mdx mice	(Jearawiriyapaisarn et al., 2008)
β-Thalassemia	β-globin	PPMO	Restoration of HbA production in erythroid cells from peripheral blood of thalassemic patients by free uptake of PMO	(Suwanmanee et al., 2002a, 2002b)
Spinal muscular atrophy (SMA)	SMN2	MOE-PS	Induction of exon 7 inclusion in human SMN2 transgenic mice	(Hua et al., 2008)
Inflammatory diseases	TNFR2	LNA	Potent and persistent anti-TNF-α effects by induction of novel splice variant Δ7TNFR2 in mice	(Graziewicz et al., 2008)
	MyD88	MOE-PS	Anti-inflammatory effects by induction of MyD88S in mice	(Vickers et al., 2006)
Cancer	HER2	MOE-PS	Triggering of apoptosis by induction of novel splice variant Δ15HER2 triggered in breast cancer cells	(Wan et al., 2008)

Dystrophia myotonica type 1	ClC-1	PMO	Induction of exon 7a skipping in ClC-1 in HSALR and Mbn1ΔE3/ΔE3 mice	(Wheeler et al., 2007)
Menkes disease	ATP7A	PMO	Rescue of Menkes phenotype by correction of aberrant ATP7A splicing in a zebrafish disease model	(Madsen et al., 2008)
Ataxia telangiectasia	ATM	PMO	Production of functional ATM protein by correction of aberrant ATM splicing in cell culture	(Du et al., 2007)
Atherosclerosis	APOB	2'OMePS	Induction of novel splice variant APOB87skip27 in cells in culture	(Khoo et al., 2007)
Propionic and methylmalonic acidemias	PCCA, PCCB, and MUT	PMO	Full recovery of PCC and MUT enzymatic activity in fibroblasts from patients	(Ugarte et al., 2007)

[a]References of the most significant publications.

Abbreviations: APOB, apolipoprotein B; ATM, Ataxia-telangiectasia mutated gene; ClC-1, chloride channel 1; MUT, methylmalonyl Coenzyme A mutase; 2'OMePS, 2'O-methyl phosphorothioate; PMO, phosphorodiamidate morpholino; PPMO, peptide-conjugated PMO; PCCA, propionyl coenzyme A carboxylase-α subunit; PCCB, PCC-β subunit; SMN2, spinal motor neron 2; TNF-α, tumor necrosis factor-α; TNFR, TNF receptor.

Table 1: Overview of potential therapeutic targets for AMOs and other splice-switching oligonucleotides (SSOs) [14]

AMOs direct pre-mRNA splicing by binding sequence elements and blocking access of the spliceosome and other splicing factors. They can be applied to (1) restore correct splicing of an aberrantly spliced transcript, (2) produce a novel splice variant that is not normally expressed, or (3) manipulate alternative splicing from one splice variant to another (Fig. 9).

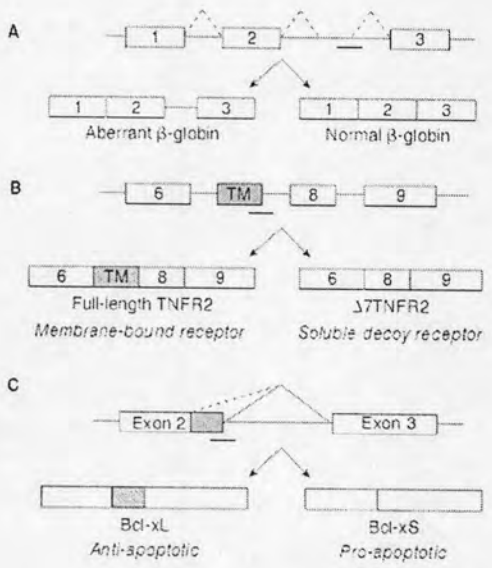

Figure 9: Application of splice-switching oligonucleotides. (A) Correction of aberrant splicing of human β-globin leads to production of functional protein -globin. (B) Production of a novel splice variant, 7TNFR2, which is a decoy receptor antagonist of TNF-α. (C) Manipulation of Bcl-x alternative splicing switches production from anti-apoptotic Bcl-xL to pro-apopototic Bcl-xS[14].