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Current Issues Regarding Prenatal Diagnosis of Inborn Errors of Cholesterol Biosynthesis

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1. Introduction

It has long been established that there is a relationship between hypercholesterolemia and cardiovascular disease in adulthood. However, only in the 90s, the biological consequence of low levels of cholesterol for mitotic cells was highlighted, with the description of the devastating effect of hypocholesterolemia on fetal development. This was supported by the discovery of a group of metabolic diseases caused by mutations in genes coding for enzymes involved in endogenous synthesis of cholesterol. This group is still growing as new diseases, phenotypes and mutated genes are being described by researchers. Despite the fact that they share some common clinical features - including abnormal morphogenesis and growth retardation - these inherited metabolic diseases are still poorly recognized in daily obstetric clinical practice.

Cholesterol is an essential lipid found in all mammalian cells. It can modulate the activity of the Hedgehog proteins, which act as morphogens that regulate the precise patterning of many embryonic structures [Gofflot et al., 2003]. Furthermore, cholesterol is a key component of lipid-rafts, which have a structural role in cellular membranes and myelin sheets and it is a precursor molecule for sterol-based compounds, including bile acids, oxysterols, neurosteroids, glucocorticoids, mineralocorticoids, and sex hormones like estrogen and testosterone [Correa-Cerro et al., 2005]. Due to the panoply of biological functions of the sterols, a decrease of its availability during pregnancy has major consequences to the fetus, severely impairing his development [Cardoso et al., 2005a].

2. Intestinal cholesterol absorption, transport and metabolism

Dietary cholesterol is absorbed from bile salt micelles, with fatty acids and phospholipids, at the proximal part of the small intestine, in a process which involves Nieman-Pick C1-Like1 protein (NPC1L1). This protein contains a sterol sensing domain (SSD) and is located in the

brush-border membrane of enterocytes where it plays a critical role in the intestinal uptake of cholesterol and phytosterols [Ikonen, 2006]. Once inside the enterocyte cholesterol is esterified by acyl-CoA: cholesterol acyltransferase (ACAT) whereas sterols other than cholesterol, are transported back into the intestinal lumen by an heterodimer transporter G5/G8 ATP binding cassette (*ABCG5*, *ABCG8*)¹. Such mechanism prevents phytosterols molecules from passing to the blood to any significant extent [Charlton-Menys & Durrington, 2007].

In order to enter in blood circulation cholesterol from enterocytes should be incorporated in lipoproteins named chylomicron. Chylomicrons assembly begins with the formation of primordial, phospholipid-rich particles in the membrane, taking together apolipoprotein B48 (*ApoB-48*)² and cholesterol. Later in the lumen of the smooth endoplasmic reticulum (ER) these particles are converted into large chylomicrons. After that, they are transported (via specialized vesicles) from the ER to the Golgi³, for secretion into the lacteals of the intestine, and then they finally pass from linfa to blood (via thoracic duct) [Hussain et al., 2005; Charlton-Menys & Durrington, 2007; Kindel, et al., 2010]. Once chilomicrons are in blood circulation they became smaller due to the action of lipoprotein lipase (LPL)⁴, which is anchored to the vascular endothelium of several organs, and hydrolyses the triglyceride from chilomicrons. Subsequently the circulating cholesterol-rich chylomicron remnant particles are uptaken by the liver in a process which involves the LDL-receptor like protein (LRP) [Charlton-Menys & Durrington, 2007].

Liver, exports exogenous and endogenous cholesterol, to tissues by VLDL lipoproteins. The biosynthesis of VLDL consists of a number of distinct stages [Hebbachi & Gibbons, 2001; Shelness et al., 2001]. Briefly, the assembly of hepatic VLDL begins inside the rough ER, where, the peptide *ApoB-100*² is synthesized at membrane bound ribosomes and then sent through a protein channel into the cytoplasm. Additionally, microsomal triglyceride transfer protein (MTP)⁵ binds the precursor peptide and joins some triglycerides, phospholipids, and cholesteryl esters, allowing *ApoB-100* to fold around a small lipid core. Then a higher amount of triglycerides are transferred into the precursor VLDL particle, and it sorts to the Golgi apparatus where additional lipids are recruited in order to form the mature VLDL lipoprotein [Daniels et al., 2009]. Finally VLDLs enter in circulation and distribute free fatty acids to muscle and adipose tissues expressing LPL and become intermediary density lipoproteins (IDLs) that can either be removed from circulation by the liver or they can lose further free fatty acids becoming low density lipoproteins (LDLs) which are important cholesterol transporters [Daniels et al., 2009].

Therefore, low density lipoprotein receptor (LDLR)⁶, a transmembrane glycoprotein responsible for uptake of cholesterol-carrying lipoproteins from blood circulation, binds lipoprotein particles at the cell surface, which are internalized by endocytosis and later in the low-pH environment of the endosome, acid-induced dissociation of ligand and receptor occurs. LDLR peptide is recycled back to the membrane and LDL particles are released into the lysosomes whose enzymes degradate the lipoproteine into amino acids and lipid components. Cholesteryl esters are hydrolyzed by lysosomal acid lipase (LAL)⁷ to free cholesterol [Daniels et al., 2009; Jeon & Blacklow, 2005]. Therefore cholesterol can be re-esterified by ACAT (a membrane-bound enzyme residing in the ER) and stored as lipid droplets [Zhang et al., 2003; Liu et al., 2005]. The mechanisms by which free and esterified cholesterol ingress and egress endosomes and lipid droplets, are not fully clarified.

Nevertheless proteins NPC1⁸ and NPC2 are involved in such process and other proteins like MLN64 (STARD3) and MENTHO (STARD3NL), are still under evaluation [Miller & Bose, 2011; Vanier, 2010].

At steady state most cholesterol is in plasma membrane, but it must move inside the cell in order to be presented to the enzymes encharged of its metabolization, and it is transported between membrane organelles (as a component of lipid bilayers) in transport vesicles, as well as by non-vesicular means [Liscum et al., 1995; Maxfield & Wüstner, 2002].

Meanwhile, the liver is consistently manufacturing high density lipoproteins (HDL) which have the critical task of removing excess cholesterol and serve as transport particles by which peripheral cell cholesterol is collected and delivered to the liver for catabolism in a process named reverse cholesterol transport [Ikonen, 2006]. The rate limiting step in this process is cholesterol efflux mediated by ABCA1⁹ [Daniels et al., 2010].

Cells inside the brain are cut off from this circuit by the blood-brain barrier and must regulate their cholesterol content in a different manner [Pfriegeer & Ungerer, 2011].

As cholesterol cannot be degraded by cells into noncyclic hydrocarbon products, hepatocytes excrete it into the bile, either directly, as free cholesterol, as well as transformed in bile salts. Bile salts are synthesized via two routes, the classic or neutral pathway and the alternative or acidic one [Kosters et al., 2003]. Cholesterol 7 α -hydroxylase (CYP7A1) is a hepatic microsomal cytochrome P450 enzyme that catalyzes the first step of bile acid synthesis in the classical pathway, whereas sterol 27-hydroxylase (CYP27A1) is the first enzyme of the alternative one. It is a mitochondrial cytochrome P450 ubiquitous enzyme with a much broader biologic role; it is involved in the 27-hydroxylation of a variety of sterols (cholesterol included) and in the formation of potentially important regulatory sterols [Dias & Ribeiro, 2011].

All major classes of biologically active steroid hormones are also synthesized from cholesterol by a complex array of enzymes located both in the mitochondria and ER [Miller, 2011; White, 1994]. Adrenals and gonads receive cholesterol from low-density lipoproteins, store it as cholesterol esters, and transport cholesterol to mitochondria by ill-defined but critical mechanisms [Miller, 2011]. Effectively, the acute quantitative regulation of steroidogenesis is determined by cholesterol import into mitochondria by the steroidogenic acute regulatory protein (STAR) which undergoes conformational changes for accept ing and discharge cholesterol molecules [Miller & Auchus, 2011]. Then P450_{scc} /CYP11A1 enzyme, located in the inner mitochondrial membrane catalyses the conversion of cholesterol to pregnenolone, the first step of steroidogenesis [Miller & Auchus, 2011].

Control of cholesterol homeostasis is a highly regulated process, consistent with the overall importance of this lipid for normal cellular function, with several transcription factors and functioning proteins playing important roles and regulating intracellular cholesterol levels [Tarling & Eduards, 2011]. Mutations in genes codifying for proteins involved in the above referred pathways (and signalled with small numbers), alter sterol homeostasis and results in specific diseases. Table 1 resumes the most commum phenotypes associated to deficient cholesterol intra and extracelular transport and metabolism which was construct based on OMIM (<http://www.ncbi.nlm.nih.gov/omim>) available data.

Disease	MIM	Gene	Phenotype
1. Sitosterolemia	#210250	<i>ABCG5, ABCG8</i>	AR. Characterized by unrestricted intestinal absorption of phytosterols. Patients show very high levels of plant sterols in the plasma with accumulation in tendons (tuberous xanthomas) and arteries.
2. Hypobetalipo- proteinaemia	+107730	<i>APOB</i>	Apolipoprotein B, occurs in the plasma in 2 main forms, apoB48 (synthesized exclusively by the gut) and apoB100 (synthesized by the liver) resulting from differential splicing of the same primary mRNA transcript. Heterozygous show reduced plasma concentrations of LDL cholesterol, total triglycerides, and APOB less than 50% of normal values.
3. Anderson disease (Chylomicrons retention disease)	#246700	<i>SAR1B</i>	AR. It is a disease of severe fat malabsorption and steatorrhea, associated with failure to thrive in infancy. Patients show low fasting plasma concentrations of plasma total, HDL, and LDL cholesterol. Electron microscopy studies of jejunal biopsy specimens showed severe steatosis, and an apparent block of chylomicron secretion from the ER into the Golgi apparatus.
4. Hyperlipo- proteinemia type Ia	#238600	<i>LPL</i>	AR. Massive hyperchylomicronemia occurs when the patient is on a normal diet and disappears completely in a few days on fat-free feeding. Caused by low tissue activity of lipoprotein lipase (a defect in removal of chylomicrons and of other triglyceride-rich lipoproteins). Characterized by attacks of abdominal pain, hepatosplenomegaly, eruptive xanthomas, and lactescence of the plasma.
Hyperlipo- proteinemia type Ib	#207750	<i>APOCII</i>	Deficiency of apolipoprotein C-II the activator of lipoprotein lipase. Clinically and biochemically simulates lipoprotein lipase deficiency.
5. Abetalipo- proteinaemia	#200100	<i>MTP</i>	Caused by mutations in the microsomal triglyceride transfer protein. Features are celiac syndrome, pigmentary degeneration of the retina, progressive ataxic neuropathy and acanthocytosis. Intestinal absorption of lipids is defective, serum cholesterol very low, and serum beta lipoprotein absent.

Disease	MIM	Gene	Phenotype
6. Familial hypercholesterolemia	#143890	<i>LDLR</i>	AD. Caused by mutations in the low density lipoprotein receptor gene. Heterozygotes develop tendinous xanthomas, corneal arcus, and coronary artery disease (fourth or fifth decade). Homozygotes develop these features at an accelerated rate in addition to planar xanthomas, which may be evident at birth in the web between the first 2 digits.
7. Wolman disease	#278000	<i>LIPA</i>	It is caused by lysosomal LAL deficiency. Homozygous present liver failure, hypercholesterolemia, hypertriglyceridaemia, liver fibrosis, early atherosclerosis and early death. Heterozygous show a milder phenotype named cholesteryl ester storage disease.
8. Niemann-Pick disease type C	#257220	<i>NPC1</i>	AR. Neurodegenerative lipid storage disorder characterized by a highly variable clinical phenotype. In the classic form symptoms appearing between 2 and 4 years and patients develop neurologic abnormalities (ataxia, grand mal seizures, and loss of previously learned speech). Diagnosis relies on detection of delayed LDL-derived cholesterol esterification on skin fibroblasts as well as in filipin staining.
9. Tangier disease	#205400	<i>ABCA1</i>	AR disorder characterized by markedly reduced levels of HDL resulting in tissue accumulation of cholesterol esters. Clinical features include very large, yellow-orange tonsils, enlarged liver, spleen and lymph nodes, hypocholesterolemia, and abnormal chylomicron remnants. Coronary artery disease is increased in heterozygotes for ABCA1 deficiency.
10. 7 α -hydroxylase deficiency	*118455	<i>CYP7A1</i>	Hypercholesterolemia (high LDL), hypertriglyceridemia, premature gallstone disease.
11. Cerebrotendinous xanthomatosis	#213700	<i>CYP27A1</i>	AR. Characterized by progressive neurologic dysfunction, premature atherosclerosis, and cataracts. Large deposits of cholesterol and cholestanol are found in tissues, particularly the Achilles tendons, brain, and lungs.
12. Congenital lipid adrenal hyperplasia	#201710	<i>CYP11A1</i> <i>STAR</i>	Congenital lipid adrenal hyperplasia is the most severe form of congenital adrenal hyperplasia. Affected individuals can synthesize no steroid hormones; hence, all are phenotypic females with a severe salt-losing syndrome that is fatal if not treated in early infancy.

Legend: AR-autosomal recessive; AD-autosomal dominant; LAL-lysosomal acid lipase

Table 1. Diseases / phenotypes associated to deficient cholesterol transport and metabolism

3. The cholesterol biosynthesis pathway

Cholesterol is a 27-carbon tetracyclic compound synthesised by all nucleated mammalian cells [Kelly & Herman, 2001] by a metabolic process involving approximately 30 enzymatic reactions [Ikonen, 2006] which take place in several cellular compartments: cytoplasm, ER (or its extensions), nuclear envelope and peroxisomes [Ikonen, 2006; Thompson et al., 1987]. The substrate for cholesterol synthesis is acetyl-CoA which is derived largely from glucose in the brain, and from fatty acids and other fuels in other tissues [Clayton, 1998].

Although complex, the biosynthesis of cholesterol is only one element of the larger isoprenoid biosynthetic system, which incorporates the *de novo* synthesis of important biomolecules as diverse as dolichol, ubiquinone, heme A or farnesyl pyrophosphate [Kelly & Herman, 2001].

For simplification one can consider five major steps on the metabolic pathway of cholesterol synthesis [Figure 1].

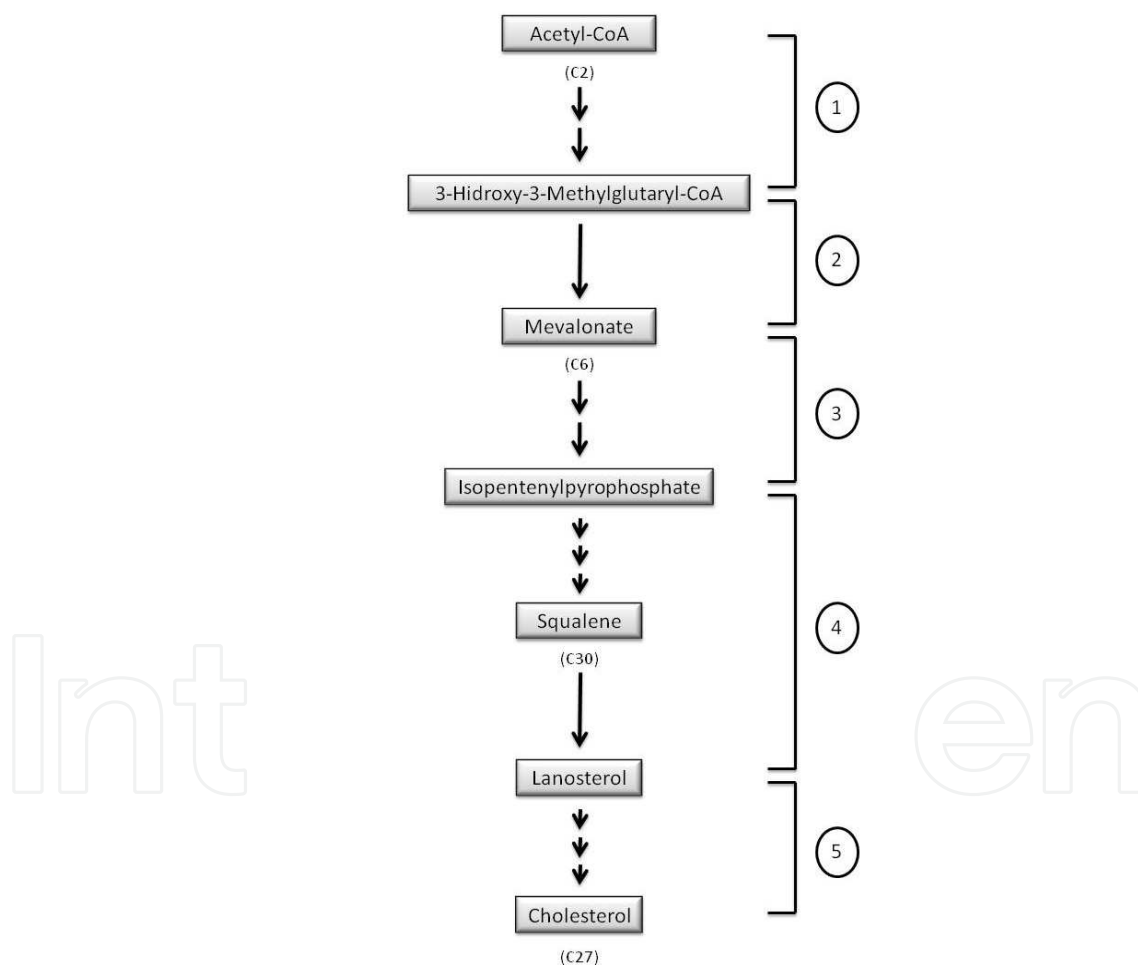


Fig. 1. Simplified schematic representation of cholesterol biosynthesis pathway.

Steps one to four catalyze the transformation of acetyl-CoA into the first sterol of the cascade: lanosterol. The fifth step includes all the reactions needed to transform this sterol into cholesterol [<http://themedicalbiochemistrypage.org/cholesterol.html>]. The biologically significant details of each step are:

1. Conversion of acetyl-CoAs to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), an organic acid conjugate of intermediate metabolism that is also important for ketogenesis.
2. HMG-CoA conversion to mevalonate by HMG-CoA reductase, the limiting step of cholesterol biosynthesis. In fact, HMG-CoA reductase is subject to complex regulatory control by four distinct mechanisms (i) feed-back inhibition, (ii) control of gene expression, (iii) rate of enzyme degradation and (iv) phosphorylation-dephosphorylation (the first three are exerted by the cholesterol molecule itself).
3. Mevalonate conversion to the isoprene based molecule, isopentenyl pyrophosphate with the concomitant loss of CO₂.
4. Isopentenyl pyrophosphate conversion to lanosterol. The reactions of this stage are also required for the synthesis of other important compounds as previously referred: (i) isopentenyl-tRNAs (the isopentenyl groups in tRNAs are thought to be important in stabilizing codon-anti-codon interaction, thus preventing misreading of the genetic code during protein synthesis), (ii) dolichol which is required for protein N-glycosylation, (iii) farnesyl and geranylgeranyl pyrophosphate essential for protein prenylation which is a post-translational modification required to commit proteins to cellular membranes and (iv) ubiquinone, which is an important component of mitochondrial respiratory chain [Clayton, 1998, Ikonen, 2006].
5. Lanosterol conversion to cholesterol [Figure 2].

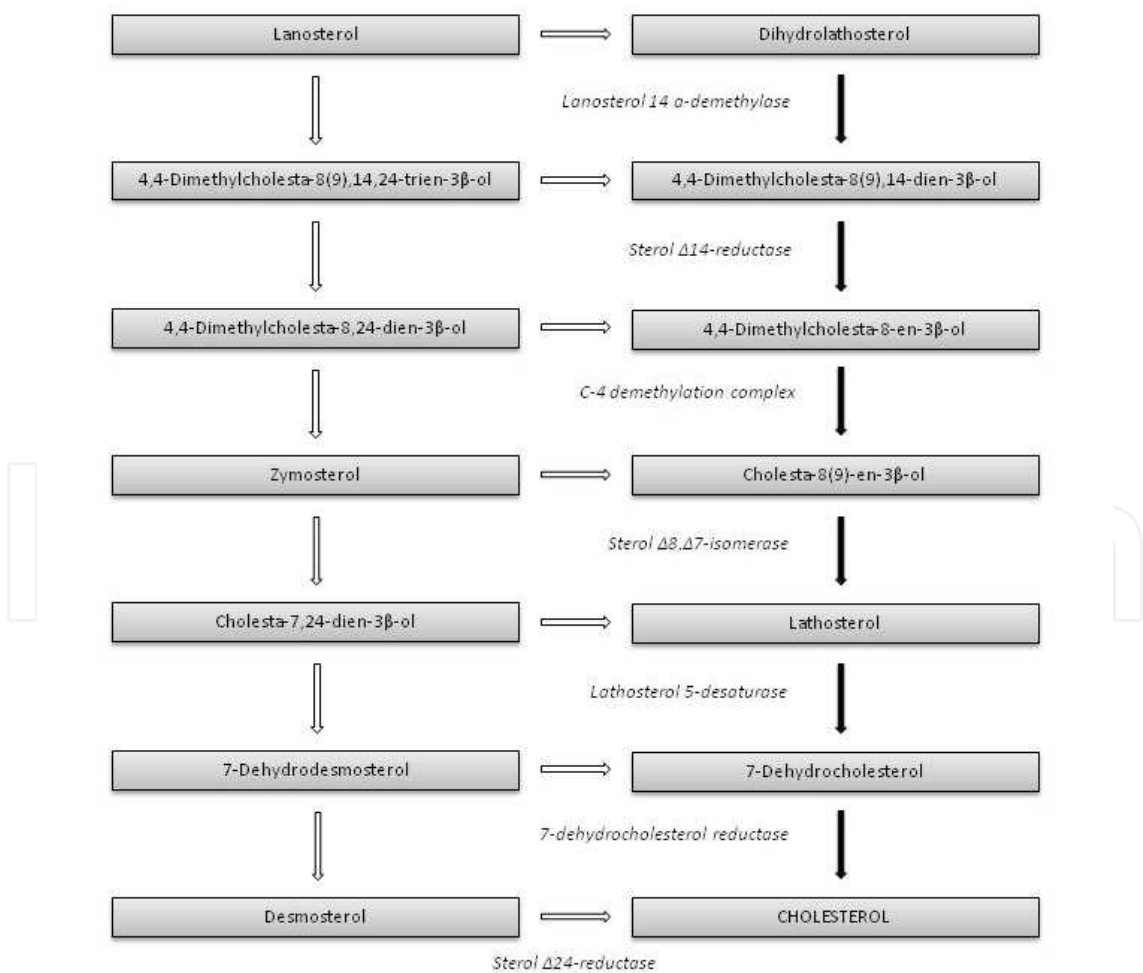


Fig. 2. The distal part of cholesterol biosynthesis pathway: from lanosterol to cholesterol

This stage constitutes the post-lanosterol part of cholesterol biosynthesis pathway. It includes a series of enzymatic reactions namely (i) three demethylations at C4 α , C4 β and C14, which converts the 30-carbon molecule of lanosterol into the 27-carbon cholesterol ; (ii) the isomerization of the $\Delta^{8(9)}$ double bond to Δ^7 double bond, (iii) one reaction of desaturation to form a Δ^5 double bond; (iv) and finally the reduction of three double bonds Δ^{14} , Δ^{24} , Δ^7 [Porter, 2003].

4. Sterols and development

Cholesterol is indispensable for embryogenesis and fetal development in higher vertebrates. The fetus obtains most cholesterol from *de novo* synthesis, with fetal sterols synthesis rates being greater than those observed in other extra hepatic tissues. This happens, most likely, because of the large cholesterol fetal requirements, in order to sustain the rapid intra-uterine growth [Woollett, 2005]. Nevertheless, the fetus appears to have an exogeneous source of cholesterol as well. In fact, some studies have suggested that maternal cholesterol may also contribute to the cholesterol accrued in the fetus [Lindegaard et al., 2008; McConihay et al., 2001; Yoshida & Wada, 2005]. Reinforcing this hypothesis, a strong association with preterm delivery in caucasian mothers with low serum cholesterol during pregnancy was found, and smaller birth weight in term babies from such mothers [Edison et al., 2007]. Thus, two layers of cells must be crossed by maternal cholesterol to reach the fetal circulation (i) the trophoblasts (which form the layer closest to the maternal circulation) and (ii) the endothelium (locate between the trophoblast and fetal circulation) [Woollett, 2011]. According to some experiments, the modulation of maternal-fetal cholesterol transport has potential for *in utero* therapy of fetuses that lack the ability to synthesize cholesterol [Lindegaard et al., 2008; Woollett, 2005].

Distal inhibitors of cholesterol biosynthesis have been studied for more than 30 years as potent teratogens capable of inducing cyclopia and other birth defects. These compounds specifically block the Sonic hedgehog (Shh) signaling pathway [Cooper et al., 1998]. Hedgehog (Hh) proteins comprise a group of secreted embryonic signaling molecules that are essential for embryonic patterning [Kolejáčková et al., 2010]. In higher vertebrates, including humans, they are implicated in an increasing number of different developmental processes. In fact, Shh proteins were implicated in neural tube development, lung and kidney morphogenesis and hair development, Shh and Indian hedgehog were related with skeletal morphogenesis and gastrointestinal development and Desert hedgehog with male differentiation, spermatogenesis and development of peripheral nerve sheaths [Waterman & Wanders, 2000]. Cholesterol has an important role in regulation and modification of Hedgehog proteins, what links cholesterol to early embryonic development. [Kolejáčková et al., 2010]. Decreasing levels of cellular sterols correlate with diminished response of the Hh signal and sterol depletion affects the activity of Smoothened, an essential component of the Hh signal transduction apparatus [Cooper et al., 2003].

Mutations in the Sonic Hedgehog gene cause holoprosencephaly and this cerebral malformation has also been associated with perturbations of cholesterol synthesis and metabolism in mammalian embryos [Gofflot et al., 2001]. Furthermore, in rodents, triparanol treatment reproduces limb defects observed in human syndromes of cholesterol biosynthesis defects by a modification of Shh signaling in the limb resulting in an imbalance

of Indian Hedgehog expression in the forming cartilage leading to reduced interdigital apoptosis and syndactyly [Gollob et al., 2003].

5. Inborn errors of post-squalene cholesterol biosynthesis

Genetic defects in enzymes responsible for cholesterol biosynthesis have recently emerged as important causes of congenital anomalies. Patients with these metabolic diseases present with complex malformation syndromes involving different organs and systems [Yu & Patel, 2005]. So far, nine polymalformative disorders due to enzymatic defects in post-squalene cholesterol biosynthesis have been identified:

- a. Smith-Lemli-Opitz syndrome (SLOS),
- b. X-linked dominant chondrodysplasia punctata type 2 (CDPX2),
- c. Congenital hemidysplasia with ichthyosiform erythroderma and limb defects syndrome (CHILD)
- d. CK syndrome
- e. Greenberg dysplasia,
- f. Antley-Bixler syndrome with ambiguous genitalia (POR deficiency)
- g. Desmosterolosis,
- h. Lathosterolosis,
- i. Sterol-C4-methyloxidase-like deficiency.

6. Prenatal diagnosis of cholesterol biosynthesis disorders

For most inborn errors of metabolism, before attempting to perform prenatal diagnosis, it is essential to establish, or confirm the diagnosis of the disorder under consideration in the proband, or affected relatives. Nevertheless as inborn errors of cholesterol biosynthesis have been associated to a gestational biochemical marker (low maternal estriol) and abnormal ultrasound features, in many cases one can suspect of this spectrum of disorders in the course of a pregnancy, even without a previous index case in the family.

6.1 Smith-Lemli-Opitz syndrome

The Smith-Lemli-Opitz syndrome (SLOS, MIM #270400), is the most frequent disease of this group of inherited metabolic disorders, with a prevalence that varies between 1: 22.000 and 1: 60.000, depending on the population. Nevertheless, a higher prevalence (between 1: 2.500 and 1: 4.444) - close to that of cystic fibrosis and higher than phenylketonuria - would be expected based on carriers frequency studies [Porter et al., 2003].

SLOS is caused by a deficit of the enzyme 7-dehydrocholesterol reductase (3- β -hydroxysterol- Δ^7 -reductase, E.C.1.3.1.21), encoded by the *DHCR7* gene located on 11q13. This enzyme catalyses the conversion of 7-dehydrocholesterol to cholesterol [Waterham & Wanders, 2000].

Since most of the cholesterol required for fetal growth and development is synthesized by the fetus, enzymatic deficiency affecting endogenous cholesterol biosynthesis leads to intrauterine growth restriction and aberrant organogenesis. Surveys of large series of patients with SLOS showed a constellation of severe abnormalities including intellectual

disability, microcephaly, failure to thrive, dysmorphic face, limb abnormalities and genital abnormalities in males [Cardoso et al., 2005b].

Prenatal diagnosis of SLOS has been performed in many pregnancies both retrospectively and prospectively. The prenatal diagnosis of this autosomal recessive metabolic disorder is based on a conjugation of methods that detect the dual aspects of the pathology: the polimalformative syndrome and the metabolic abnormalities [Cardoso et al., 2005a].

6.1.1 Ultrasound findings

A number of fetuses have been so far signaled as suspected of SLOS due to the identification, by ultrasound, of suggestive fetal abnormalities, such as: nuchal edema, microcephaly, cleft palate, polydactyly, cystic kidneys, ambiguous genitalia or a 46, XY karyotype in a phenotypically female fetus [Johnson et al., 1994, Kelley & Hennekam, 2001]. Nevertheless there is no pathognomonic ultrasound pattern associated with SLOS [Irons & Tint, 1998].

Concerning the detection of malformations in SLOS fetus, Goldenberg and collaborators evaluated the main abnormalities identified. Their results are as follows i) in the first trimester [11-13 weeks of gestational age]: increased nuchal translucency (26%); ii) in the second trimester [20-22 weeks of gestational age]: nuchal edema (26%), kidney malformation (26%), polydactyly (10%) [Figure 3], ambiguous genitalia (6%) [Figure 3], cerebral malformation (10%), heart malformation (10%), intrauterine growth restriction (20%); iii) in the third trimester [30-34 weeks of gestational age]: intrauterine growth restriction (46%) [Goldenberg et al., 2004]. However one should be aware that prenatal US examination of affected fetuses can also be normal [Irons & Tint, 1998].

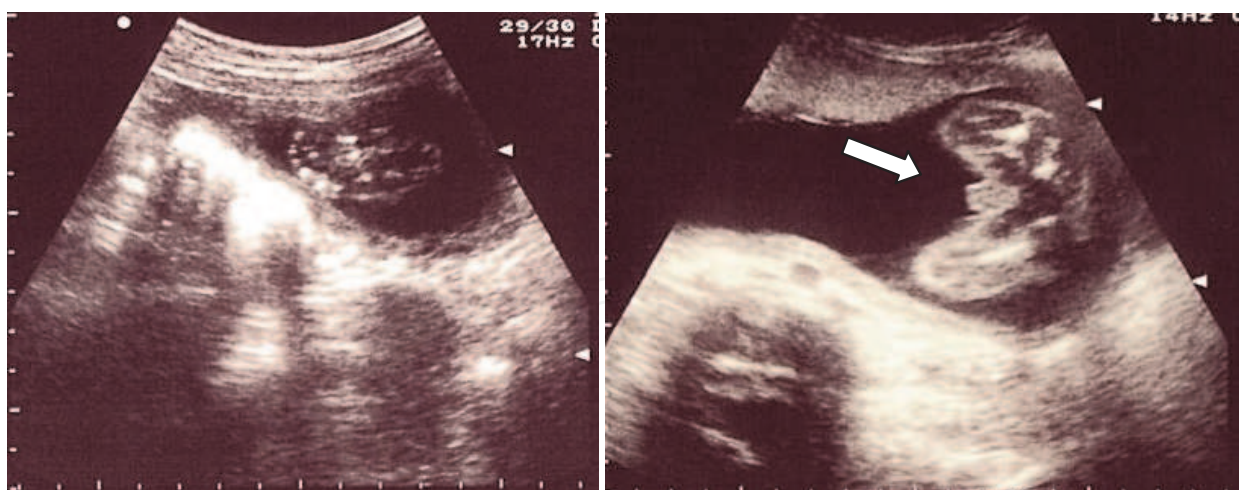


Fig. 3. Two ultrasound images of a male fetus with SLOS. The white arrows are pointing a left foot with postaxial polydactyly (on the left) and a penis with large root and hypospadias (on the right).

6.1.2 Serum maternal markers

The fetoplacental biosynthesis of free estriol (μE_3) requires cholesterol as substrate [Palomaki et al., 2002]. Thus unconjugated estriol is produced by the fetus and then crosses

the placental barrier entering into mother's blood circulation. By consequence fetal hypocholesterolemia can be suspected, if the pregnant woman shows low levels of this compound in serum. Low or undetectable unconjugated estriol levels in maternal serum and amniotic fluid have been reported in pregnancies of fetus affected with SLO [Hyett et al., 1995; Rossiter et al., 1995; Kratz & Kelley, 1999; Cardoso et al., 2005a; Craig et al., 2006].

Free estriol is not a specific marker of SLO and several other causes of low unconjugated estriol are known, namely intrauterine fetal demise and steroid sulphatase deficiency [Irons & Tint, 1998]. Meanwhile algorithms were proposed based on available values of maternal serum α -fetoprotein and human chorionic gonadotrophin together with free estriol (obtained on the second-trimester screening program, for Down syndrome and open neural tube defects) which provide a more accurate estimation of individual risk for SLOS [Palomaki et al., 2002; Craig et al., 2006; Craig et al., 2007].

According to our and others experience [Cardoso et al., 2005a; Dubuisson et al., 2008] a low level of free estriol alone is not a robust indicator for testing a pregnancy for SLOS. However the association of i) abnormal fetal US with ii) normal fetal karyotype and iii) low levels of unconjugated estriol on maternal blood are highly suggestive [Cardoso et al., 2005a; Shinawi et al., 2005; Dubuisson et al., 2008].

6.1.3 Biochemical approach

The first report concerning prenatal diagnosis SLOS based on biochemical profile of sterols in amniotic fluid was performed in 1995 by Abuelo and collaborators [Abuelo et al., 1995]. Since then several cases referring the quantification of 7-dehydrocholesterol in amniotic fluid as well as in chorionic villus either by gas chromatography or gas chromatography-mass spectrometry have been published [Irons & Tint, 1998; Chevy et al., 2005; Cardoso et al., 2005a]. The diagnosis can also be made based on the detection of low enzymatic 7-dehydrocholesterol reductase activity on cultivated amniocytes or chorionic villus [Linck et al., 2000; Ginat et al., 2004].

Nowadays liquid chromatography-tandem mass spectrometry (LC-MS-MS) is available in many clinical biochemistry laboratories and efforts have been made in order to apply this highly sensitive technology to the diagnosis of inborn errors of cholesterol biosynthesis. Recently a protocol for prenatal diagnosis of SLOS by LC-MS-MS became available [Griffiths et al., 2008]. Another promising approach concerns the non-invasive SLOS biochemical prenatal diagnosis based on identification and measurement of abnormal steroids in maternal urine [Jezela-Stanek et al., 2006; Shackleton et al., 2007].

6.1.4 Mutation analysis of *DHCR7* gene

Prenatal diagnosis of SLOS by mutation analysis of *DHCR7* gene on DNA extracted directly from amniotic fluid, chorionic villus or the respective cell cultures is widespread used at this time [Yu & Patel, 2005]. This approach is accurate and reliable [Loeffler et al., 2002]. Moreover molecular prenatal diagnosis should be considered an option: (i) in laboratories without facilities for biochemical analysis, (ii) in families with known mutations who are interested in early and rapid testing and (iii) in cases with ambiguous biochemical results [Nowaczyk et al., 2001; Löffler et al., 2009; Waye et al., 2007].

6.2 X-linked dominant chondrodysplasia punctata type 2, CHILD Syndrome and CK Syndrome

X-linked dominant chondrodysplasia punctata type 2 (CDPX2 or Conradi-Hunermann-Happle syndrome, MIM #302960) and Congenital hemidysplasia with ichthyosiform erythroderma and limb defects syndrome (CHILD syndrome, MIM #308050), are two X-linked dominant skeletal dysplasias caused by hypocholesterolemia, which affect almost exclusively females, as they are typically lethal in hemizygous males. In both diseases affected females usually present at birth with skeletal and skin abnormalities [Herman et al., 2000]. In CDPX2 these include epiphyseal stippling and asymmetric rhizomelic shortening of the limbs, follicular atrophoderma, ichthyosiform erythroderma (following the Blaschko's lines), asymmetrical cataracts and accumulation in plasma and body tissues of cholesterol precursors: 8-dehydrocholesterol and cholest-8(9)-en-3 β -ol. The disorder is caused by a deficiency of the enzyme 3 β -hydroxysterol- Δ^8, Δ^7 -isomerase encoded by *EBP* gene [Braverman et al., 1999]. In most severe cases ultrasound during pregnancy can show polyhydramnios, intra-uterine growth restriction or both [Kelley et al., 1999]. Only a few prenatal diagnosis have been reported and they were based on the identification of mutations on *EBP* gene.

Both CHILD syndrome and CK syndrome are caused by mutations in *NSDHL* gene encoding a 3 β -hydroxysterol dehydrogenase [Konig et al., 2000, McLaren et al., 2010]. CHILD is associated with an inflammatory nevus with unique lateralization, ipsilateral hypoplasia of the body that affects all skeletal structures including shortness or absence of limbs and viscera such as lungs, heart or kidneys [Happle et al., 1980]. CK syndrome is an X-linked recessive intellectual disability syndrome characterized by dysmorphism, cortical brain malformations, asthenic build, increased methyl-sterol levels and it is associated to hypomorphic temperature-sensitive alleles [McLaren et al., 2010]. As far as we know, there are no reports on prenatal diagnosis of these two syndromes in literature.

6.3 Greenberg dysplasia

The Greenberg dysplasia (MIM #215140), is a rare and lethal autosomal recessive skeletal dysplasia characterized by hydrops fetalis, ectopic calcifications, "moth-eaten" skeletal dysplasia, short limbs, and abnormal chondro-osseous calcification [Madazli et al., 2001]. In 2003, Waterham and collaborators found elevated levels of a cholesterol precursor (cholesta-8,14-dien-3 β -ol) in cultured skin fibroblasts of a fetus with Greenberg dysplasia, that were compatible with a deficiency of the enzyme 3- β -hydroxysterol- Δ^{14} reductase from the cholesterol biosynthesis pathway. Sequencing analysis of two candidate genes encoding putative human sterol- Δ^{14} reductases allow the identification of an homozygous mutation (resulting in a truncated protein) in *LBR* gene (which encodes a bifunctional protein – lamin B receptor – with both lamin B binding and sterol- Δ^{14} reductase domain) [Waterham et al., 2003]. After the publication of these results it was acknowledged that Greenberg dysplasia was a disorder of cholesterol biosynthesis. Nevertheless, recent studies with mice models raised some doubts about this classification and proposed that Greenberg dysplasia should be classified as a laminopathy rather than an inborn error of cholesterol synthesis [Wassif et al., 2007]. Despite this controversy i) ultrasound, ii) biochemical analysis of sterols on cultivated skin fibroblasts and iii) mutation analysis of *LBR* remain available to diagnose Greenberg dysplasia prenatally.

6.4 Antley–Bixler

Antley-Bixler syndrome (ABS) is a very rare congenital multiple malformation disorder characterized by craniofacial anomalies, skeletal defects and, in a subset of patients, ambiguous genitalia. The craniofacial anomalies include brachycephaly due to severe craniosynostosis. The typical facial dysmorphism include: depressed nasal bridge with very short upturned nose, small mouth and dysplastic ears. Skeletal features include radiohumeral or other forearm synostoses, arachnodactyly, bowing of femurs, multiple contractures and neonatal fractures. A variety of congenital heart defects and gastrointestinal malformations have also been reported. Urogenital anomalies include absent, dysplastic, ectopic, or horseshoe kidneys, abnormal ureters and abnormalities of the external genitalia, including cryptorchidism. [Porter et al., 2011]. Recently it has been considered that the minimum criteria to establish the diagnosis of ABS are the presence, from the prenatal period, of craniosynostosis and radiohumeral synostosis [McGlaughlin, et al., 2010].

ABS is genetically heterogeneous and there has been some debate about the definition of the disease: by the clinical phenotype versus according to the genetic etiology [Cragun & Hopkin, 2005; Huang et al., 2005]. In fact, some patients present ABS without disordered steroidogenesis (MIM #207410) due to one gain-of-function mutation in the *FGFR2* gene (hence following autosomal dominant inheritance) while others present ABS with disordered steroidogenesis (MIM #201750) due to two loss of function mutations in the *POR* gene (therefore, following autosomal recessive inheritance) [Flück et al., 2004]. ABS patients with mutations in *POR* are included in the spectrum of metabolic disorders due to abnormal cholesterol pathway. The *POR* gene encodes a cytochrome P450 oxidoreductase. The POR protein is an electron donor to many cytoplasmic P450 enzymes, including the cholesterol C14-lanosterol demethylase encoded by the *CYP51* gene and several other enzymes involved in steroid hormone synthesis.

ABS patients due to POR deficiency usually present with abnormal genitalia (underdeveloped genitalia and cryptorchidism in affected 46,XY males and external virilization, with clitoromegaly and fused labia, in 46,XX females) but a much wider spectrum of the remaining phenotype (milder craniofacial and skeletal malformations and normal cognitive function). The mildest end of the *POR* spectrum presents as individual with only steroidogenesis defects (amenorrhea, polycystic ovarian syndrome and infertility) [Fukami et al., 2009].

Independently of the severity, all POR deficient patients demonstrate biochemical evidence of partial blocks at multiple steps in the conversion of cholesterol to cortisol, estrogens, and androgens and biochemical diagnosis of POR deficiency can be made by GC-MS of urinary steroids, which reveals a characteristic profile of elevated pregnenolone and 17-OH-progesterone and other progesterone metabolites, in the presence of low androgens. Mineralocorticoid synthesis and metabolism are normal. Mild abnormalities of serum steroids are often, but not always, present. The steroid metabolites that accumulate in POR deficiency are consistent with partial deficiencies of 21-hydroxylase (*CYP21A2*) and 17 α -hydroxylase (*CYP17A1*). The biochemical findings are explained by the fact that the POR enzyme serves as an electron donor for all cytoplasmic P450 enzymes, including *CYP17A1* and *CYP21A2*.

A POR deficient fetus can be referred to prenatal diagnosis due to common abnormalities such as oligohydramnios or two vessel cord, to fetus with full blown ABS (multiple craniofacial, skeletal and urogenital abnormalities as described in detail above) on the ultrasound. Moreover, the diagnosis of ABS due to POR deficiency should be considered in pregnancies with low or undetectable serum uE3, like in SLOS [Cragun et al., 2004; Williamson et al., 2006]. Likewise, maternal virilization (e.g. acne, facial edema) shouldn't be overlooked as it may comprise an important clue for the diagnosis of POR deficiency [Cragun et al., 2004].

There should be an effort towards the confirmation of the diagnosis through molecular analysis since it is clinically relevant to distinguish ABS patients with disordered steroidogenesis from ABS patients with normal steroidogenesis, not only because of differences in inheritance patterns, but also because patients with POR deficiency are vulnerable to different risk factors and require different management.

6.5 Possible approaches for prenatal diagnosis of Desmosterolosis, Lathosterolosis and Sterol-C4-methyloxidase-like deficiency

Desmosterolosis (MIM #602398) and Lathosterolosis (MIM #607330) and Sterol-C4-methyloxidase-like deficiency are additional autosomal recessive polimalformative syndromes due to defective cholesterol biosynthesis. Desmosterolosis is associated to mutations in *DHCR24* gene and 3- β -hydroxysterol- Δ^{24} -reductase deficiency [Clayton et al. 1996, Waterham et al., 2001] whereas mutations on *SC5D* gene, that encodes lathosterol-5-desaturase, cause lathosterolosis [Krakowiak et al., 2003, Brunetti-Pierri et al., 2002]. Both diseases are still poorly characterized due to the small number of cases identified. They both share some phenotypic characteristics with SLOS and the diagnosis of new cases will contribute for a better phenotypic characterization of these metabolic disorders. In order to contribute for the recognition of these entities, we have recently established reference values for several sterols in amniotic fluid at different gestational ages (lathosterol and desmosterol included) [Amaral et al., 2010].

A patient with psoriasiform dermatitis, arthralgias, congenital cataracts, microcephaly, and developmental delay was recently identified as harboring mutations in sterol-C4-methyl oxidase-like gene (*SC4MOL*), which encodes a sterol-C4-methyl oxidase. This enzyme also belongs to the cholesterol biosynthesis pathway and catalyses the demethylation of C4-methylsterols. Sterol-C4-methyl oxidase deficiency is a novel disease of inborn errors of cholesterol biosynthesis, which clinical spectrum remains to be defined [He et al., 2011].

No prenatal diagnosis of these three disorders has so far been reported. However, in theory, it can be performed based on sterols profile of amniotic fluid or through the identification of mutations in the above mentioned genes.

7. The teratogenic effect of drugs that interfere with cholesterol biosynthesis

Many women on reproductive age take medicines on a regular basis. However, most drugs presently available on the market are not licensed for use in pregnancy. Therefore, these women may conceive on medication, leading to a large number of early pregnancies being exposed to a wide range of drugs. Moreover, fetuses have been increasingly exposed to new

classes of compounds, as these compounds have been shown to be effective and well tolerated outside pregnancy [Kyle, 2006].

Drugs that block crucial steps of cholesterol biosynthesis exert a teratogenic effect on the fetus, mimicking the genetically determined enzymatic defects. These phenocopies have features that resemble the phenotype of the corresponding inherited metabolic disease. Drugs with such properties are identified among antifungal, hypocholesterolemic and some antineoplastic agents.

7.1 Antifungals

A variety of antimycotic compounds are currently available to treat systemic or mucocutaneous fungal infections and some of them are capable of penetrating the placental barrier [Moudgal & Sobel, 2003]. Azoles antifungals act by competitive inhibition of CYP51 (lanosterol 14 α -demethylase) decreasing the synthesis of ergosterol, the main sterol in fungal cell membrane. Apart from ergosterol depletion, selective inhibition of CYP51 also leads to accumulation of lanosterol and other 14-methylsterols, resulting in alterations of fungal wall, cell growth, cell replication and inhibition of morphogenic transformation of yeasts into mycelia [Giavini & Menegola, 2010, Pursley et al., 1996]. The inhibitory potential of these compounds is not limited to fungi; it has also been seen in a number of mammalian cytochrome P450-dependent activities, including microsomal enzymes [Sheets & Mason, 1984] and studies carried out in pregnant animals taking high doses of azole fungicides revealed their teratogenic potential. Malformations were found at branchial apparatus (related with facial structures), axial skeleton and limbs [Giavini & Menegola, 2010, Menegola et al., 2003].

Fluconazole, a bis-triazole anti-fungal agent is commonly used to treat human mycosis. It shows excellent oral absorption, low plasma protein affinity, long half-life, high concentrations in urine and CSF, minimal adverse reactions, wide spectrum of anti-fungal activity and it has high specificity for fungal cytochrome P450 system [Agrawal et al., 1996].

Nevertheless, at least five cases reporting children with a multiple malformation syndrome due to first-trimester fluconazole exposure were published. In all cases, high doses (400-800 mg/day) of fluconazole were administered during several weeks (in order to treat a severe systemic mycotic infection) before women were aware that they were pregnant [Aleck & Bartley, 1997; Lopez-Rangel & Van Allen, 2005; Pursley et al., 1996]. The newborns showed anomalies analogous to those seen in experimental animals [Aleck & Bartley, 1997] and the phenotype identified resembled that of Antley-Bixler syndrome (thoroughly described above) [Lopez-Rangel & Van Allen, 2005]. A possible explanation for the similarity between this embryopathy and Antley-Bixler phenotype is a compromised cytochrome P450 system in both situations.

In contrast to the above described teratogenicity of fluconazole, the use of topical azoles for treatment of superficial fungal infections in pregnancy seems safe and efficient [Moudgal & Sobel, 2003] and there are several epidemiologic reports of tens of women who took sporadically low doses (50 -150mg/day) of fluconazole during first trimester of pregnancy and did not show increased overall risk of birth defects, compared with a control group (fluconazole free during pregnancy) [Giavini & Menegola, 2010; Inman et al., 1994; Mastroiacovo et al., 1996; Nørsgaard et al., 2008]. Hence, as it has been previously

demonstrated for other drugs [Polifka et al., 2002], dosage seems to be a critical factor on the teratogenicity potential of fluconazole given that apparently the exposure is harmful only if it is chronic or exceeds a certain threshold.

7.2 Statins

Another group of compounds that strongly interferes with cholesterol biosynthesis pathway are the hypocholesterolemic drugs: statins. These compounds inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase that catalyses a limiting step of cholesterol biosynthesis: the conversion of HMG-CoA to mevalonate. Because this key compound is also required for the synthesis of several other biologically important molecules, levels of HMG-CoA reductase may regulate many cellular processes and functions in cell. In fact, knock out embryos for HMG-CoA reductase died prematurely suggesting that the loss of HMG-CoA reductase activity leads to implantation failure or to embryonic death prior to implantation [Ohashi, 2003].

The use of statins for treatment of hyperlipidemia is increasingly common [Taguchi et al., 2008] and the effectiveness of these agents in reducing mortality and morbidity associated with coronary artery disease is well established [Kusters et al., 2010]. However, when pregnancy is considered, lipid-lowering drugs are often discontinued because of the fear of teratogenic effects [Kusters et al., 2010].

There is scarce and conflicting evidence on the teratogenic potential of statins and most of the information on drug safety for the fetus is limited to animal studies, a few case reports and retrospective uncontrolled data [Avis et al., 2009]. A study focusing on the toxicity of atorvastatin in pregnant rats and rabbits has shown that only the highest doses tested - which were also toxic for the mothers - had harmful effects in pregnancy by increasing postimplantation loss and decreasing fetal body weight [Dostal et al., 1994]. Later, studies evaluating *in vitro* effects of statins in a human placental model have demonstrated that simvastatin (i) inhibited half of the proliferative events in the villi, (ii) increased apoptosis of cytotrophoblast cells and (iii) significantly decreased secretion of progesterone from the placental explants. These effects may contribute to the failure of implantation and be deleterious to the growth of the placental tissues which could explain the higher abortion rate and teratogenicity observed in animals exposed to statins during pregnancy [Kenis et al., 2005].

Moreover, due to the occurrence of unplanned pregnancies, there are a number of cases in which statins were inadvertently taken during the first trimester of pregnancy, some of them resulting in newborns with birth defects [Edison & Muenke, 2004, 2005; Petersen et al., 2008; Trakadis et al., 2009]. Nevertheless, these studies are not conclusive due to ascertainment bias: (i) in some cases pregnant women took potentially teratogenic drugs other than statins [Trakadis et al., 2009] or (ii) previous maternal health disorders like pre-pregnancy diabetes, obesity or both [Petersen et al., 2008] or (iii) the small number of cases identified enables the validation of a statistically significant conclusion [Edison & Muenke, 2004, 2005; Petersen et al., 2008]. Furthermore, none of the studies evaluated the possibility of decreased fertility, increased pre-implantation or peri-implantation losses that could be increased as shown in animal experiments [Elkin & Yan, 1999; Lee et al., 2007; Ohashi et al., 2003; Richards & Cole, 2006; Zapata et al., 2003].

7.3 Tamoxifen

Another interesting compound is tamoxifen, a nonsteroidal selective estrogen receptor modulator, used for the treatment and prevention of breast cancer [de Medina et al., 2004]. This drug is currently used as adjuvant treatment in premenopausal women affected or at risk for breast cancer [Berger & Clericuzio, 2008]. Tamoxifen has also been reported to protect against the progression of coronary artery diseases in human and animal models [de Medina et al., 2004]. Such property can be related to the capacity of tamoxifen to inhibit several enzymes related with cholesterol metabolism namely acyl-CoA cholesterol acyltransferase which catalyses cholesterol esterification [de Medina et al., 2004] as well as with its hypocholesterolemic properties. As a matter of fact, tamoxifen inhibits several cholesterologenic enzymes, namely: (i) sterol Δ -8-isomerase, (ii) sterol Δ -24-reductase, (iii) sterol Δ -14-reductase, and the administration of such compound to humans and laboratory animals results in a drastic reduction in cholesterol and a marked accumulation of certain sterol intermediates in serum [Cho et al., 1998].

Despite tamoxifen long use in clinical practice, its teratogenic potential remains inconclusive. Furthermore, while the evidence of effects of tamoxifen in humans in utero is minimal, animal studies have shown evidence of teratogenicity (abnormalities of genital tract and irregularly ossified ribs in rat pups) and delayed vaginal opening in female offspring of guinea pigs [Barthelmes & Gateley, 2004; Berger & Clericuzio, 2008]. According to a review of seven papers referring tamoxifen prenatal exposure there was (i) one case of ambiguous genitalia after 20 weeks exposure to a daily dose of 20 mg (ii) one case of Goldenhar's syndrome after 26 weeks exposure to 20 mg/day (with simultaneous exposure to other teratogenic drugs during the first 6 weeks) [Barthelmes & Gateley, 2004]. Later, one case of Pierre Robin sequence (small mandible, cleft palate and glossoptosis) associated with first trimestre fetal exposure was also published in a pregnancy with gestational diabetes [Berger & Clericuzio, 2008].

If we put together the fact that (i) this drug was initially developed as a contraceptive agent [Barthelmes & Gateley, 2004], (ii) it inhibits several enzymes of cholesterol biosynthesis pathway, (iii) in most cases the exposure to tamoxifen occurs very early – sometimes even before women are aware of the pregnancy – and (iv) no adverse effects were observed in 85 cases in which tamoxifen was taken as preventive drug of breast cancer (without association to other potentially teratogenic compounds exposure), we are lead to the conclusion that tamoxifen has a „all-or-none“ effect. In other words, exposure to tamoxifen may cause affected embryos that are lost very early (even before women are aware of the pregnancy) or fetal survival without any malformation.

All in all, one should highlight the role of the medical appointments on the evaluation of the interaction of drugs with the developing fetus. Ideally, pregnancies should be prepared in advance and, at that time, the potentially teratogenic drugs should be replaced by less harmful medicines or, if possible, discontinued. After conception and when evaluating a fetus with malformations on the ultrasound, doctors should bear in mind the role of teratogenic drugs that can produce phenocopies of genetically determined disorders.

8. Conclusion

During the last two decades inborn errors of cholesterol biosynthesis have emerged as a group of metabolic disorders that should be included in the differential diagnosis of

intrauterine growth restriction and abnormal embryogenesis as well as in the investigation of the etiology of low levels of maternal estriol in the second trimester of pregnancy.

Patients with these metabolic diseases present with complex malformation syndromes involving different organs and systems. As metabolic pathways are biological interactive networks, one specific blockage activates new routes for detoxication of accumulated products followed by excretion. For example, it was noticed that urine from pregnant women at risk for SLOS revealed abnormal steroids derived from 7-dehydrocholesterol. One can assume this as a general rule, and postulate that, in defective cholesterol biosynthesis accumulated sterols are metabolized originating “new” steroids. If quantification of abnormal steroids in maternal urine (which is non invasive and easy to perform) becomes widely available, one can envision a future in which prenatal diagnosis of inborn errors of cholesterol biosynthesis is extended to most fetuses with developmental abnormalities.

Furthermore it is possible that, as it is being developed in other fields of medical genetics, high throughput technologies might also be used in the setting of metabolic disorders: for example, a microarray chip with oligonucleotide probes targeted to all the genes involved in metabolic pathways and the application of a next generation sequencing platform to perform sequencing analysis of those genes. This would allow for the identification of both copy number variants and point mutations of the genes implicated in the inborn errors of cholesterol biosynthesis pathway, thus promoting a global and thorough approach to these diseases, a better phenotype-genotype correlation and a more accurate knowledge of the spectrum of these disorders.

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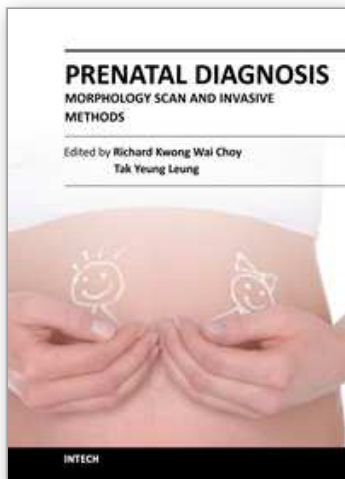
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This book provides detailed and comprehensive coverage on various aspects of prenatal diagnosis-with particular emphasis on sonographic and molecular diagnostic issues. It features sections dedicated to fundamentals of clinical, ultrasound and genetics diagnosis of human diseases, as well as current and future health strategies related to prenatal diagnosis. This book highlights the importance of utilizing fetal ultrasound/clinical/genetics knowledge to promote and achieve optimal health in fetal medicine. It will be a very useful resource to practitioners and scientists in fetal medicine.

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