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Disorders of Cholesterol Metabolism and Their Unanticipated Convergent Mechanisms of Disease

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Abstract

Cholesterol plays a key role in many cellular processes, and is generated by cells through de novo biosynthesis or acquired from exogenous sources through the uptake of low-density lipoproteins. Cholesterol biosynthesis is a complex, multienzyme-catalyzed pathway involving a series of sequentially acting enzymes. Inherited defects in genes encoding cholesterol biosynthetic enzymes or other regulators of cholesterol homeostasis result in severe metabolic diseases, many of which are rare in the general population and currently without effective therapy. Historically, these diseases have been viewed as discrete disorders, each with its own genetic cause and distinct pathogenic cascades that lead to its specific clinical features. However, studies have recently shown that three of these diseases have an unanticipated mechanistic convergence. This surprising finding is not only shedding light on details of cellular cholesterol homeostasis but also suggesting novel approaches to therapy.

THE CELL BIOLOGY OF CHOLESTEROL

The Evolution and Functions of Sterols

Sterols are a group of primarily unsaturated solid steroid alcohols found in the membranes of all eukaryotic cells. Phylogenetically distinct organisms synthesize characteristic sterols; for example, cholesterol, ergosterol, and phytosterol are the predominant sterols found in terrestrial vertebrates, fungi, and plants, respectively. The evolution of sterols has been the subject of much debate because they are absent from prokaryotes [although rare exceptions have been documented (64)] but are ubiquitously expressed in eukaryotes (82). One hypothesis to explain this phylogenetic divide is that sterol evolution was driven by the increase in atmospheric oxygen levels, which coincided with the prokaryote/eukaryote transition (16, 35). Consistent with this idea, sterol biosynthesis is dependent entirely on oxygen (82): The biosynthesis of one molecule of cholesterol consumes 11 molecules of O_2, and the biosynthesis of one molecule of ergosterol consumes 12 molecules of O_2 (106, 117). Sterols have also been proposed to serve as oxygen sensors in yeast (22, 50), which may have been another selective pressure that drove their evolution. For the remainder of this article, we focus on the mammalian sterol cholesterol, reviewing the complexities associated with cholesterol-related inherited metabolic disorders and their novel unanticipated interrelationships.

Cholesterol

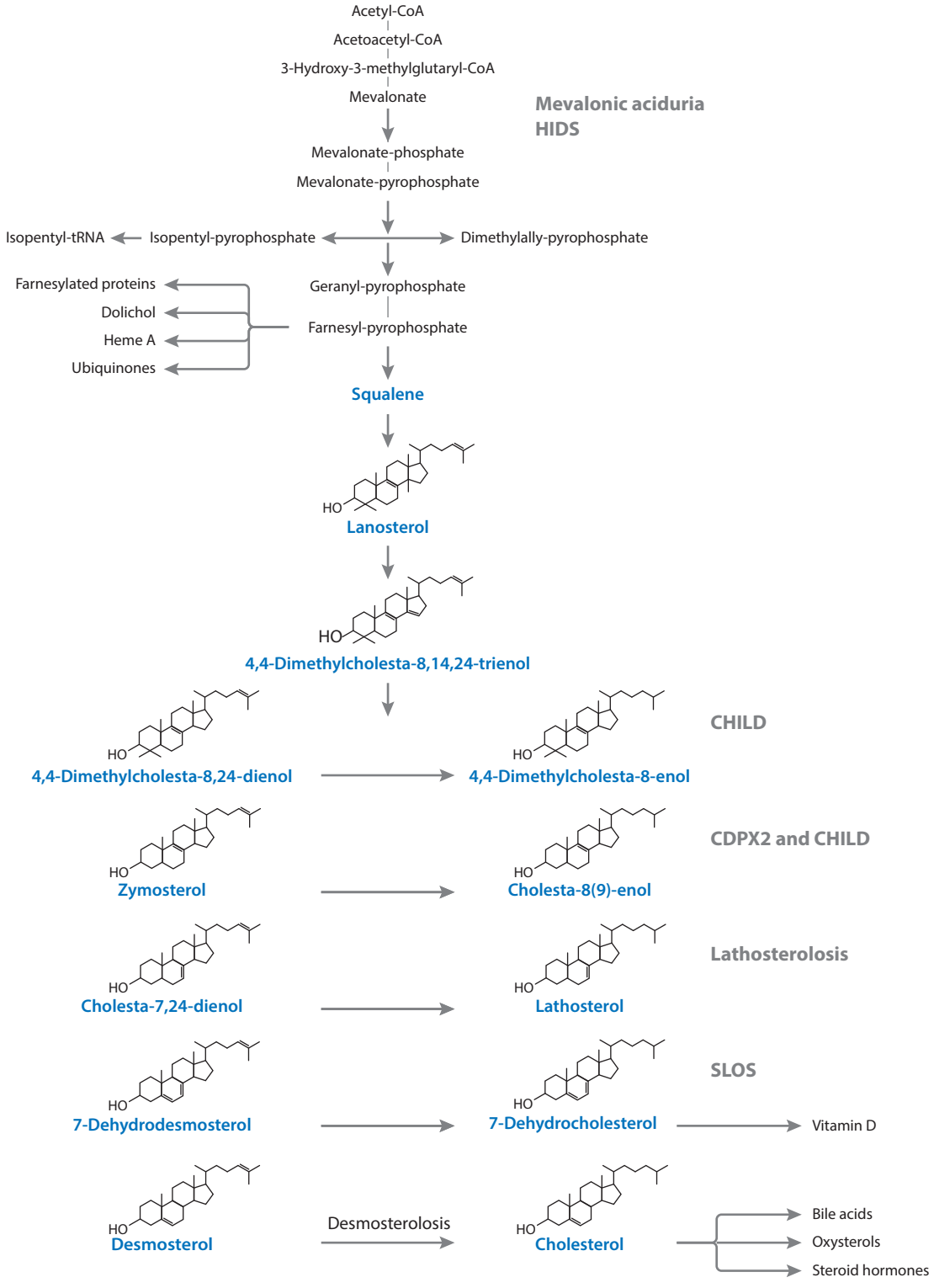
Cholesterol was first isolated from gallstones and has been intensively studied since its discovery in pre-revolutionary France (36). It is a highly regulated amphipathic lipid (37) that plays important roles in a variety of homeostatic systems (25, 36, 51, 74, 88, 105, 115). Cholesterol is essential for the normal growth and development of mammals and in membranes, where it regulates membrane fluidity and is a key constituent of lipid rafts (95, 103). These dynamic signaling platforms are implicated in multiple cellular processes, but their physiological relevance is still the subject of debate (66, 79, 103). Cholesterol is also essential for myelin formation (57, 127) and in developmental signaling via the hedgehog pathway (10, 105), in which active hedgehog proteins are covalently modified with cholesterol. Sterols also play multiple other roles in this signaling pathway (10, 105). In addition, there is growing evidence that sterols and sphingolipids are coregulated, although the details of the underlying mechanisms and their biological significance remain to be fully elucidated (41, 42).

In addition to functions in membrane biology, cholesterol and its biosynthetic intermediates serve as key metabolic precursors for the synthesis of corticosteroids, vitamin D, bile acids (107), and steroid hormones, including neurosteroids (5, 26, 121) (**Figure 1**). These in turn interact with nuclear receptors (e.g., the FXR, PXR, and VDR nuclear receptors for bile acids and the estrogen, androgen, progestin, glucocorticoid, and mineralocorticoid nuclear receptors for steroids), thereby regulating other aspects of cell function (141). With the exception of the liver and steroidogenic tissues, mammalian cells do not metabolize cholesterol but instead modulate cholesterol content in their membranes by regulating cholesterol biosynthesis, uptake, and export from the cell via ATP-binding cassette (ABC) transporters (91).

In view of the complex regulation and diverse functions attributable to sterols, it is perhaps not surprising that inherited defects in genes involved in cholesterol metabolism lead to several

Figure 1

Cholesterol biosynthesis and associated diseases. Abbreviations: CDPX2, X-linked dominant chondrodysplasia punctata type 2 (Conradi–Hünermann–Happle syndrome); CHILD, congenital hemidysplasia with ichthyosiform nevus and limb defects syndrome; HIDS, hyperimmunoglobulinemia D syndrome; SLOS, Smith–Lemli–Opitz syndrome.


Acetyl-CoA
Acetoacetyl-CoA
3-Hydroxy-3-methylglutaryl-CoA
Mevalonate
Mevalonic aciduria
HIDS
Mevalonate-phosphate
Mevalonate-pyrophosphate
Isopentyl-tRNA
Isopentyl-pyrophosphate
Dimethylally-pyrophosphate
Farnesylated proteins
Dolichol
Heme A
Ubiquinones
Geranyl-pyrophosphate
Farnesyl-pyrophosphate
Squalene
HO
Lanosterol
4,4-Dimethylcholesta-8,14,24-trienol
4,4-Dimethylcholesta-8,24-dienol
4,4-Dimethylcholesta-8-enol
CHILD
Zymosterol
Cholesta-8(9)-enol
CDPX2 and CHILD
Cholesta-7,24-dienol
Lathosterol
Lathosterolosis
7-Dehydrodesmosterol
7-Dehydrocholesterol
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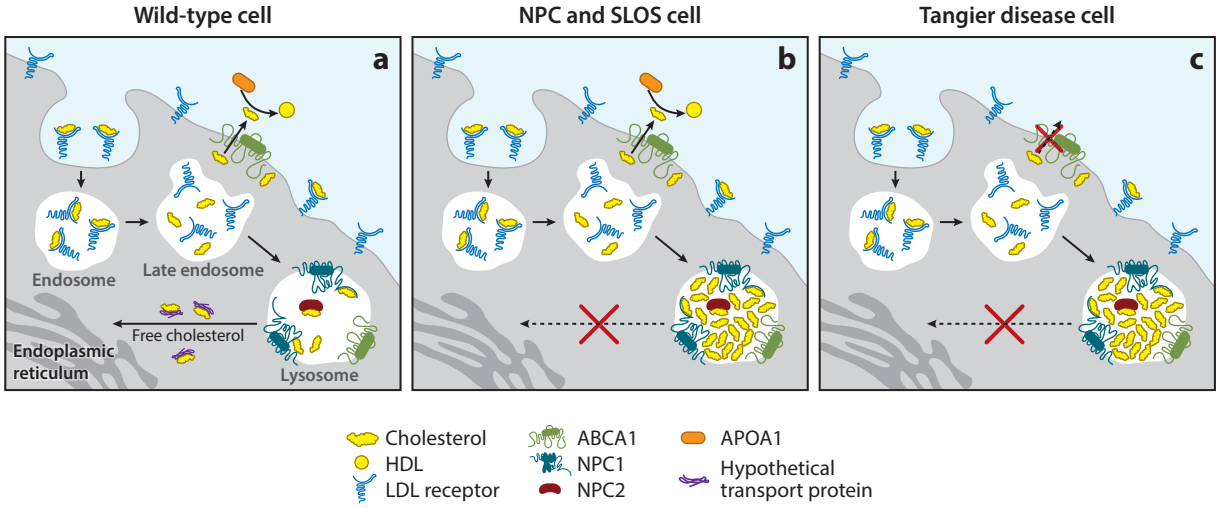

Figure 2

Cellular consequences of Niemann–Pick disease type C (NPC disease), Smith–Lemli–Opitz syndrome (SLOS), and Tangier disease for cholesterol homeostasis and the common involvement of the NPC pathway: (*a*) wild-type cell, (*b*) NPC and SLOS cell, and (*c*) Tangier disease cell.

particularly severe and complex human diseases (100). Before discussing these diseases, we review aspects of cholesterol homeostasis to provide a framework for understanding the consequences of cholesterol metabolism/transport defects in human disease.

Sources of Cholesterol

Most mammalian cells can acquire cholesterol from two independent sources. The first is de novo biosynthesis by a multienzyme-catalyzed pathway (**Figure 1**); the second is the uptake of exogenously derived cholesterol associated with plasma low-density lipoprotein (LDL) from the circulation (**Figure 2*a***). The balance between these two pathways depends on cell type and the availability of LDL-derived cholesterol. The de novo biosynthetic pathway can be manipulated pharmacologically using statins, which inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (**Figure 1**). There are inherited diseases associated with defects in both pathways (de novo synthesis and exogenous uptake/intracellular trafficking), and here we briefly review what is known about each pathway to provide a context for understanding the unexpected links between the different diseases involving defects in cholesterol homeostasis.

Cholesterol Biosynthesis

All nucleated cells have the capacity to generate cholesterol de novo, with the liver being the most significant source. Cholesterol biosynthesis occurs in two distinct stages: the presqualene pathway and the postsqualene pathway (82). The presqualene pathway contributes to sterol and isoprenoid synthesis, whereas the postsqualene pathway is essential for cholesterol and vitamin D biosynthesis (**Figure 1**). Cholesterol contains 27 carbons, all of which are derived from acetyl-CoA (82). The first step in the pathway involves the condensation of three acetates to form a 6-carbon intermediate, mevalonate. The next step converts mevalonate to activated isoprenes, which are then polymerized to form a 30-carbon linear molecule, squalene. Squalene then cyclizes to form the classical four-ring steroid nucleus. The final generation of cholesterol involves oxidation reactions

and methyl group modifications (82). **Figure 1** schematically details the cholesterol biosynthetic pathway.

LDL-Derived Cholesterol

LDL-derived cholesterol originates primarily from dietary sources via the liver. The major route by which exogenously derived cholesterol is taken up into cells is through the LDL receptor (LDLR) (**Figure 2*a***). Additional receptors that can mediate the uptake of modified LDL include scavenger receptors (76, 116), but for the purposes of this review, we focus exclusively on the LDLR pathway.

The significance of LDLR was first demonstrated through the pioneering work of Goldstein & Brown (38), who were studying familial hypercholesterolemia. Müller (78) originally described familial hypercholesterolemia in 1938 as an autosomal dominant trait, with affected individuals exhibiting high levels of cholesterol in their blood that resulted in myocardial infarctions at a relatively early age. Khachadurian (58) later described two forms of the disease: the severe homozygous form and the milder heterozygous form. Goldstein & Brown (38) demonstrated the existence of LDLR and found that it is internalized by a clathrin-dependent mechanism (1, 2). They also discovered that the cholesterol taken up via this route mediates key regulatory functions, including feedback inhibition of cholesterol biosynthesis (11). Yamamoto et al. (139) subsequently showed that a defect in the gene encoding LDLR causes familial hypercholesterolemia.

This is an excellent example of how the study of a rare inherited metabolic disease identified a fundamental cellular pathway and, furthermore, provided the framework for the concept of receptor-mediated endocytosis (1). LDL-derived cholesterol undergoes a complex intracellular trafficking itinerary that facilitates its utilization by the cell. The details of how cholesterol traffics within cells and, in particular, how it leaves the lysosome remain the subject of investigation.

Cholesterol Trafficking

Most cells express cell-surface LDLR that binds apolipoprotein B (ApoB) in the phospholipid layer of LDL particles (11, 139). LDLR can also recognize apolipoprotein E (ApoE) in chylomicron remnants and very-low-density lipoprotein (VLDL) remnants (116). The cholesterol-rich particles bound by LDLR are internalized via clathrin-coated vesicles (2). As the endosomes acidify, LDL dissociates from its receptor, and LDLR recycles back to the plasma membrane for reutilization (116). When free LDL reaches the late endosome/lysosome compartments, acid lipase hydrolyzes cholesterol esters (116). The free cholesterol generated is then available for transport to other sites in the cell, e.g., the plasma membrane, mitochondria, and endoplasmic reticulum (ER).

There is evidence that the Rab11 GTPase is involved in the vesicular transport of cholesterol to the plasma membrane and that MLN64 facilitates cholesterol movement to steroidogenic mitochondria (15). However, how cholesterol reaches the ER remains unclear. One possibility is that it involves direct transfer via lysosome:ER contact sites. Another possibility is that cholesterol is effluxed from lysosomes by the action of a cholesterol transporter to an unidentified sterol transfer protein located in the cytosol or on the cytosolic face of the limiting membrane of the lysosome. Two lysosomal storage diseases, Niemann–Pick disease types C1 and C2 (NPC1 and NPC2, respectively), provided support for a potential role of a cholesterol transport pathway (125). Both diseases involve the storage of multiple lipids (cholesterol and sphingolipids) in peripheral tissues and the brain, with cholesterol a prominent storage lipid in non–central nervous system (CNS) tissues such as the liver (125). The NPC1 protein is a multimembrane-spanning protein localized to the limiting membrane of the late endosome/lysosome (14), whereas the NPC2 protein

is a soluble mannose-6-phosphate-targeted cholesterol-binding protein that is found in the lysosol (81) and had also previously been found at high levels in epididymal fluid, where it was first described (and where it is instead termed HE1) (65).

One hypothesis is that NPC2 transfers cholesterol to NPC1, which then facilitates its egress from the lysosome through an unknown mechanism (53). NPC1 or NPC2 deficiency causes accumulation (storage) of unesterified cholesterol in the late endosome/lysosome, preventing its delivery to the ER and subsequent esterification. This in turn leads to impaired regulation of cholesterol homeostatic genes, including those encoding LDLR and HMG-CoA reductase, and impaired oxysterol generation (34). Paradoxically, NPC disease therefore has features of both storage and deficiency (129). NPC disease is discussed in more detail below.

Reverse Cholesterol Transport

Cholesterol can leave cells by a process termed reverse cholesterol transport (89). ABC transporters, particularly the ubiquitously expressed ABCA1, are the key players in this process (119). Inherited defects in ABCA1 lead to Tangier disease (85) (discussed in detail below). When *Abca1* is knocked out in mice, virtually no high-density lipoprotein (HDL) is detectable in the circulation, illustrating that the function of ABCA1 is a prerequisite for HDL formation and the maintenance of circulating HDL levels (40).

Tangier patients are also characterized by low plasma HDL levels (48, 59). The primary apolipoprotein of HDL is apolipoprotein A-1 (ApoA-1); when ApoA-1 binds to ABCA1, it triggers cholesterol and phospholipid efflux via a poorly understood mechanism (119). These lipids are then transferred to ApoA-1 to form discoidal HDL particles (**Figure 2*a***). ABCG1 and ABCG4 then transfer additional cholesterol to nascent HDL and to other acceptors (115, 126). Lecithin:cholesterol acyltransferase, an enzyme found in plasma, esterifies cholesterol, leading to the maturation to globular HDL particles (115). ABCA1 traffics between the late endocytic system and the plasma membrane and has been implicated in late endocytic trafficking (83, 84).

Cholesterol Homeostasis: The Role of Active Cholesterol

One unanswered question is how cells sense cholesterol levels and how they regulate cholesterol levels in their membranes. New evidence has recently emerged that focuses attention on active, or free, cholesterol. It has been known for many years that plasma membrane sterols complex with polar lipids, such as sphingolipids (6, 88). Once the binding capacity of the polar lipids is saturated, the excess uncomplexed cholesterol is in an active, or free, state (115). These active molecules are dispersed in the membrane and have an increased ability to spontaneously escape the membrane or be chemically modified. This suggests that, in this active state, cholesterol is exposed from the membrane and thus accessible to acceptor proteins (115).

Experiments that increased plasma membrane cholesterol levels revealed enhanced transfer of cholesterol to other membranes or increased extractability by β-cyclodextrins. This suggests the intriguing possibility that basal cholesterol levels in cells are equivalent to the total binding capacity of cholesterol in a given membrane, such that there is minimal active cholesterol. If excess active cholesterol is present, it equilibrates down a concentration gradient, leading to changes in homeostatic gene expression. The point of this model is that cells sense not total cholesterol levels but rather the fraction above the binding capacity of the membrane lipids. Experimental evidence in support of this model has been recently reviewed (115).

Van Meer et al. (123) proposed that in reverse cholesterol transport, the function of the ABC transporters (such as ABCA1) is to increase the exposure of cholesterol at the plasma membrane

(not to fully efflux it) in order to facilitate collisional transfer to protein acceptors. This is supported by the observation that the addition of ceramide to membranes displaces cholesterol and stimulates ABCA1-mediated cholesterol efflux, whereas cholesterol efflux is decreased when sphingomyelin levels are elevated (115).

Cholesterol Metabolism in the Brain

Because diseases of cholesterol metabolism frequently present with CNS pathology, it is important to consider some of the unique aspects of brain cholesterol metabolism. Unesterified cholesterol is an important component of the plasma membrane in all cells, but it is present at particularly high levels in brain cells. It is a major component of compact myelin, a specialized form of the plasma membrane of oligodendrocytes. Brain cholesterol accounts for some 23% of the sterol content of the mammalian body, even though the brain is only approximately 2% of total body weight (25). It is distributed between myelin and the plasma membranes of neurons and glia.

The main barrier to the movement of metabolites into or out of the CNS is the blood–brain barrier. Studies using a variety of experimental approaches in multiple species have found no evidence of cholesterol moving from the plasma into the CNS, even during development, when sterol levels in the brain undergo dramatic expansion (25). Instead, cholesterol is synthesized locally within the brain and, most important, the differential rates of synthesis mirror rates of sterol accumulation in regions of the brain measured (25). Although neurons can synthesize cholesterol, they require a significant additional source of cholesterol for their function, which is derived primarily from glial cells (25, 44, 73, 94). The details of cholesterol metabolism in the brain remain relatively poorly understood; however, it is known that cholesterol egress from the CNS into the plasma does occur, but only in the form of 24-hydroxy cholesterol (25).

DISORDERS OF CHOLESTEROL BIOSYNTHESIS

Among the approximately 7,000 inborn errors of metabolism is a family of diseases that result from defects in genes involved in sterol metabolism. Here, we focus on disorders of postsqualene cholesterol biosynthesis. All of these disorders are rare; Smith–Lemli–Opitz syndrome (SLOS), which was recognized in 1993 as the prototypic cholesterol biosynthesis disorder (54, 120), is the most common, with an average incidence of 1 in 50,000 live births. Many of these syndromes have corresponding mouse models, some of which are spontaneous mutants and others of which have been generated by genetic manipulation. Interestingly, all of these disorders, although distinct, have overlapping phenotypes, suggesting some common pathological mechanisms. Here, we provide a brief clinical description of each disease; for additional details, we refer readers to the recent review by Porter & Herman (100).

Antley–Bixler Syndrome

Patients with Antley–Bixler syndrome (OMIM #207410) present with severe craniofacial abnormalities; skeletal defects, including radiohumeral synostosis; and, frequently, ambiguous genitalia (100). There is debate about whether this disorder's pathology is due to impaired cholesterol synthesis or impaired steroidogenesis. The gene mutated in this disorder, *POR*, encodes a cytochrome P450 oxidoreductase, which acts as an electron donor to numerous P450 enzymes. One of these enzymes is the cholesterogenic C14 lanosterol demethylase. Dysregulation of this enzyme leads to the storage of 4,4-dimethylcholesta-8(9),14-dien-3β-ol and 4,4-dimethylcholesta-8(9),14,24-trien-3β-ol.

Hydrops-Ectopic Calcification–Moth-Eaten Skeletal Dysplasia

Hydrops-ectopic calcification–moth-eaten skeletal dysplasia (OMIM #215140), also referred to as Greenberg dysplasia, is a lethal skeletal dysplasia that may be a disorder of cholesterol biosynthesis, although there is debate about whether it is actually a laminopathy because of the dual role of lamin B receptor (LBR) (130). It was initially noted to be associated with trace elevations of 14-diene-3β-ol and cholesta-8(9),14,24-trien-3β-ol (**Figure 1**). The accumulation of these sterols indicated a defect at the level of the Δ14 sterol reductase. Interestingly, this is the only enzymatic step in this region of the pathway in which redundancy exists, i.e., the Δ14 sterol reductase (TM7SF2, SR-1) and LBR. The first case was identified as a homozygous mutation in *LBR* (134), and a subsequent study identified eight additional cases (90).

Congenital Hemidysplasia with Ichthyosiform Nevus and Limb Defects Syndrome and CK Syndrome

Congenital hemidysplasia with ichthyosiform nevus and limb defects syndrome (CHILD syndrome; OMIM #308050) and CK syndrome are two distinct but related disorders. Because both disorders are caused by mutations in the NADH steroid dehydrogenase-like gene (*NSDHL*), they are commonly referred to as NSDHL-related disorders. CHILD syndrome presents as an X-linked male-lethal syndrome, whereas CK syndrome presents as an X-linked recessive disorder.

NSDHL is part of a three-enzyme complex responsible for the C4 demethylation of the sterol ring structure. Elevated levels of 4,4-dimethylcholesta-8-en-3β-ol and 4,4-dimethylcholesta-8,24-en-3β-ol (**Figure 1**) have been reported, with the highest aberrant sterol concentrations detected in cultured fibroblasts from the affected skin lesions grown in lipoprotein-deficient media. The traditional clinical presentation of CHILD syndrome is the presence of unilateral skin lesions and ipsilateral limb reductions (46).

A second member of the C4 demethylation complex, sterol C4 methyloxidase, was found to be disrupted in an autosomal recessive fashion in one human patient (45). The patient presented with low serum cholesterol and elevated levels of 4α-methyl-5α-cholest-8(9)-en-3β-ol; dihydrolanosterol; 4α-methyl-5a-cholest-7(8)-en-3β-ol; 4,4′-dimethyl-5α-cholesta-8(9)-en-3β-ol; 4,4′-dimethyl-5α-cholesta-8(9)-en-3β-ol; and 4,4′-dimethyl-5α-cholesta-8(9),24-dien-3β-ol, all of which is consistent with impairment of this complex. Clinical presentation began at age two, with ichthyosiform erythroderma around the patient's umbilicus that later progressed to cover her whole body except for her palms.

Conradi–Hünermann–Happle Syndrome

Conradi–Hünermann–Happle syndrome (OMIM #302960), also called X-linked dominant chondrodysplasia punctata type 2 (CDPX2), is a second X-linked disorder that was also thought to be male lethal, although there have been reports of affected males presenting with mutations that preserve some enzymatic function (75). The disorder is caused by mutations in the gene encoding emopamil-binding protein (EBP). Although named for its ability to bind emopamil, a drug developed as a calcium channel blocker, this protein is in fact the sterol Δ7–Δ8 isomerase. As would be expected, mutations in the *EBP* gene lead to increased levels of cholesta-8(9)-en-3β-ol and zymosterol (**Figure 1**).

In females, the clinical manifestations of this disorder predominantly involve the skin and skeleton. The skeletal findings include rhizomelic shortening, epiphyseal stippling, short stature,

and scoliosis, and the skin phenotype presents as hyper- or hypopigmentation and dry, scaly skin that frequently follows the lines of X inactivation.

Lathosterolosis

Lathosterolosis (OMIM #607330) is an autosomal recessive disorder caused by mutations in the gene encoding sterol 5-desaturase (SC5D). SC5D is responsible for converting lathosterol to 7-dehydrocholesterol (7DHC) (**Figure 1**), which it does by desaturating the bond between C5 and C6 of the B ring of cholesterol, generating a double bond (**Figure 1**).

Only four patients with this disorder have been reported in the literature. The first case was originally misdiagnosed as a case of atypical SLOS with nonneuronal mucolipidosis (12). Subsequent biochemical and molecular testing revealed elevated levels of lathosterol and a homozygous missense mutation, p.Y46S, which confirmed the correct diagnosis of lathosterolosis (60). Two other patients were siblings (12), and the fourth was recently reported (47). All four patients presented with multiple malformations, several of which are also present in SLOS (see below), such as ptosis, congenital cataracts, anteverted nares, micronathia, postaxial polydactyly, ambiguous genitalia, cutaneous toe syndactyly, and cognitive impairment (47). Interestingly, under certain growth conditions, cultured fibroblasts from the first three patients developed lamellar lysosomal inclusions similar to those seen in NPC disease.

Targeted disruption of the *Sc5d* gene in mice generated a mouse model of this disease (60). Consistent with the human disorder, these mice had elevated levels of lathosterol, craniofacial malformations, and postaxial polydactyly, and fibroblasts derived from the mice mimic the lamellar lysosomal inclusions seen in the human lines (60). There is no specific therapy for this disease.

Desmosterolosis

Desmosterolosis is a rare autosomal recessive disorder of cholesterol biosynthesis caused by mutations in the gene encoding 3β-hydroxysterol Δ24-reductase (DHCR24) (19). This side-chain reduction is thought to occur at many places within the Kandutsch–Russell cholesterol biosynthetic pathway (**Figure 1**). These mutations lead to an increase in desmosterol levels in all tissues.

Three cases of desmosterolosis have been published, all with divergent clinical presentations (3, 19, 29, 111, 135). DHCR24 was initially cloned as selective Alzheimer disease indicator 1 (seladin-1). DHCR24/seladin-1 has two distinct roles in the cell: one in the ER, where it functions as a member of the cholesterol biosynthetic machinery, and one in the cytoplasm and nucleus, where it functions as a hydrogen peroxide scavenger, protecting the cell from oxidative stress (61). More patients will need to be identified to determine whether mutations in one region of the gene result in one phenotype as opposed to the other.

Pharmacological models of desmosterolosis have been generated using either U18666A or triparanol (7, 30). Interestingly, varying the dose of U18666A generates models of two distinct but potentially related disorders: desmosterolosis and NPC disease. However, the phenotype of the desmosterolosis mouse model does not replicate that of the *Npc1*$^{-/-}$ mouse model. As with lathosterolosis, there is currently no specific therapy for this disease.

Smith–Lemli–Opitz Syndrome

SLOS (OMIM #270400) is the prototypic disorder of cholesterol biosynthesis, first described in 1964 by Smith, Lemli & Opitz (113). SLOS is biochemically characterized by the abnormal accumulation of 7DHC (54) (**Figure 1**). The causative gene defect is in the gene encoding 7-dehydrocholesterol reductase (DHCR7), which functions to reduce 7DHC and generate

cholesterol in the final step of the Kandutsch–Russell biosynthetic pathway (99) (**Figure 1**). Three groups independently cloned *DHCR7* in 1998, and mutations within the gene were proven to be the molecular basis for SLOS (28, 120, 131, 136).

SLOS is more common than lathosterolosis and desmosterolosis, with an estimated incidence of between 1 in 25,000 and 1 in 60,000 (99). The carrier rate for this disease is high in the general population, but this does not lead to the high frequency of cases that would then be predicted based on this rate (87). This is because, at the severe end of the spectrum, SLOS can be a lethal disorder with major congenital anomalies, and it may account for a significant number of miscarriages in the general population (87). Mild cases combine minor physical stigmata with behavioral and learning disabilities. Typical physical manifestations include second- and third-toe syndactyly, microcephaly, micrognathia, cleft palate, polydactyly, cardiac malformations, pyloric stenosis, and genital malformation (4, 86, 102). Advancements in the clinical management of this disorder have increased the life expectancy of patients; however, there are currently few data to predict additional clinical issues that may arise later in the lives of these patients.

Currently, the only treatment for SLOS is dietary cholesterol supplementation (118). Although there is anecdotal evidence that cholesterol supplementation benefits growth by improving the overall general health of individuals with SLOS and reducing 7DHC serum levels, cholesterol therapy has significant limitations (99). The elevation of 7DHC levels persists, which is not without consequence. 7DHC may have toxic effects, and it substitutes for cholesterol within various membranes (39) and processes, such as oxysterol production, bile acid formation (80), membrane raft formation, and steroid production (72, 112). One significant limitation of cholesterol therapy is the inability of cholesterol to cross the blood–brain barrier in any appreciable amount (24). Treating the brain in SLOS is of paramount importance because SLOS individuals present with a myriad of behavioral and learning deficits, including autistic characteristics (23, 33).

Three mouse models of SLOS have been generated. The first two are null mutations, one of which deletes exons 3–5 (133) and the second of which deletes exons 4–8 (128). Both of these models recapitulate the biochemical phenotype of SLOS patients, presenting with elevated 7DHC levels and decreased cholesterol levels in all tissue and serum (20). These effects are most prominent in the CNS owing to the closure of the blood–brain barrier to cholesterol early in embryonic development (71). The peripheral organs do not reach as high a ratio of dehydrocholesterol (the combination of 7DHC and 8DHC) to cholesterol, as a result of some maternal cholesterol being supplied by the yolk sac and additional small amounts of cholesterol transported across the placenta (55). The mice present with cleft palate, an abnormal suck–swallow response caused by impairment of the *N*-methyl-D-aspartate receptors, intrauterine growth retardation, and reduced mobility (128, 133), and animals from both models die within 24 hours.

The third model is a hypomorphic knock-in mouse (20) in which the human T93M mutation has been replicated in the mouse *Dhcr7* gene. At birth, these mice also mimic the human syndrome, with elevated levels of dehydrocholesterol and a mild reduction in cholesterol. These mice are viable and reproduce. They present with second- and third-toe syndactyly (the most common physical finding in SLOS) as well as minor growth retardation, and approximately one-third develop ventricular dilatation by three months of age (20).

DISORDER OF CHOLESTEROL TRAFFICKING

Niemann–Pick Disease Type C

NPC disease is an autosomal recessive lysosomal storage disorder, a feature of which is cholesterol mistrafficking (125). Free cholesterol is stored in the late endosome/lysosome with minimal escape

of cholesterol from the acidic compartment to the ER. Besides the defect in cholesterol transport, several other lipid species are stored, including glycosphingolipids (GSLs), sphingomyelin, and sphingosine (generated from ceramide catabolism in the late endosome/lysosome) (124). In addition to this biochemical complexity, NPC disease is unusual in that it is caused by mutation in either of two independent genes: *NPC1* or *NPC2* (14, 81).

NPC disease occurs at a combined frequency of 1 in 120,000 live births, with approximately 95% of cases resulting from mutations in the *NPC1* gene (125). Apart from a small group who die within the first months of life from hepatic or pulmonary failure, most patients present with neonatal cholestatic jaundice, which usually resolves spontaneously. Hepatosplenomegaly occurs in some cases. Relentless neurodegeneration then dominates the clinical course of the disease, leading to cerebellar ataxia, dysarthria, dysphagia, dementia, and premature death, typically around the end of the second decade of life (52). Juvenile- and adult-onset variants also occur (125).

NPC1 encodes a 13-transmembrane-spanning protein of the limiting membrane of late endosomes/lysosomes, whereas *NPC2* encodes a soluble lysosomal cholesterol-binding protein (138). At the cell biological/biochemical level, two key features of this disease make it unique: It has a complex profile of lipid storage, and late endosome:lysosome fusion is profoundly impaired (125). This disorder has therefore brought to light a previously unknown cell biological pathway, the NPC pathway. As mentioned above, because unesterified cholesterol is stored in NPC disease and the NPC1 and NPC2 proteins bind and exchange cholesterol, the prevailing view is that the primary function of this pathway is to facilitate cholesterol egress from the lysosome to the ER. An alternative hypothesis is that NPC1 functions in lysosomal sphingosine transport (69). When NPC1 is inactivated in healthy cells, the first metabolite to accumulate is sphingosine (69). Sphingosine is generated in the lysosome from the catabolism of ceramide via the action of acid ceramidase. It is protonated at acidic pH and requires a transporter to leave the lysosome. The sphingosine derived from sphingolipid catabolism in the lysosome either enters the sphingolipid salvage pathway or becomes phosphorylated to generate sphingosine-1-phosphate, a key prosurvival signaling molecule (43). There is evidence in the NPC1 mouse and in NPC patients that sphingosine-1-phosphate-dependent cell lineages, such as natural killer cells, are altered in this disease (114).

Sphingosine storage has another profound effect on cells, which is to directly or indirectly cause a defect in the filling of the acidic compartment with calcium (69). The lysosome is a regulated calcium store that is uniquely mobilized via the endogenous second messenger NAADP (18, 77). Fusion and vesicular trafficking in the endocytic pathway are calcium-dependent processes, and the calcium is derived from the lysosomal compartment itself (69). Failure to release sufficient calcium in NPC disease leads to a block in trafficking/fusion essential for the functioning of the endosomal/lysosomal system, causing the secondary storage of cholesterol, GSLs, and sphingomyelin (69). The temporal relationship between the multiple metabolites stored in NPC disease suggests that cholesterol is a secondary storage metabolite in this disorder and is not central to triggering the pathogenic cascade (69, 70).

DISORDER OF REVERSE CHOLESTEROL TRANSPORT

Tangier Disease

Fredrickson (31, 32) reported the first case of Tangier disease in 1961, when he examined two siblings from Tangier Island, located in Chesapeake Bay in the United States. Both patients had typical symptoms now associated with the disease: orange-colored tonsils; enlarged spleen,

liver, and lymph nodes; and decreased HDL cholesterol levels (31, 101). Although orange-colored tonsils have been described as the presenting symptom in almost all children with Tangier disease, peripheral neuropathy is a common presenting symptom in adults (101). Tangier disease has been reported in approximately 100 patients worldwide (101) and is caused by mutations in the gene encoding ABCA1 (104, 108). Patients have little or no circulating HDL and accumulate cholesterol, leading to the formation of foam cells, an early marker of atherosclerosis; they also often develop cardiovascular disease later in life (101, 110).

The ABC transporters are the largest known membrane transport family, consisting of 49 members divided into seven subfamilies, A through G (49, 122). The membrane-associated protein ABCA1, which is defective in Tangier disease, regulates cellular cholesterol and phospholipid homeostasis by functioning as a cholesterol efflux pump (68, 109). ABCA1 mediates the transfer of lipids across the plasma membrane to apolipoproteins (ApoA-1 in particular) to form HDL particles (59)—hence the low levels of HDL in Tangier disease patients. In addition to regulating cholesterol efflux, ABCA1 has been proposed to have anti-inflammatory functions (68).

ABCA1 expression is regulated at multiple levels throughout the body, with the highest protein expression found in the liver, brain, adrenal glands, and macrophage foam cells. Interactions between apolipoproteins and ABCA1 activate multiple signaling pathways, including the JAK/STAT, protein kinase A (PKA), and protein kinase C (PKC) pathways (68). The C terminus of ABCA1 contains a PDZ domain responsible for mediating protein–protein interactions in addition to a VFVNFA motif (13). Several mutations in the ABCA1 C-terminal domain have been identified in Tangier disease patients, suggesting that it has a crucial function. Deletion of the VFVNFA domain also results in diminished ApoA-1 binding and lipid efflux (13). Tangier disease patients have structurally abnormal late endocytic vesicles in addition to impaired motility and trafficking, which are also observed in the cells of patients with NPC disease (8). Studies have shown that although ABCA1 and NPC1 may interact in the cell, the NPC1 protein is not required for the delivery of late endosome/lysosome cholesterol to ABCA1 to form HDL (8). Additionally, the ABCA1 transporter may convert pools of lipids that otherwise might associate with NPC1 into pools that can associate with ApoA-1 to form HDL particles (84).

The majority of NPC patients have low HDL cholesterol, suggesting diminished ABCA1 activity (17). NPC is the first disease known to have low HDL levels as a consequence of impaired ABCA1 regulation rather than a mutated ABCA1 protein, as seen in Tangier disease (17). The sequestration of cholesterol in the late endosome/lysosome in NPC disease, which leads to impaired sterol-response gene expression, is likely responsible for a failure to upregulate ABCA1 despite the storage of cholesterol (8). Interestingly, cholesterol mobilization by ABCA1 is critically dependent on NPC2 but not NPC1 function (9). There is currently no specific treatment for patients with Tangier disease.

UNEXPECTED MECHANISTIC LINKS BETWEEN SMITH–LEMLI–OPITZ SYNDROME, NIEMANN–PICK DISEASE TYPE C, AND TANGIER DISEASE

As discussed above, SLOS, NPC disease, and Tangier disease are three unique inherited disorders involving very different defects in cholesterol homeostasis: SLOS is the prototypic disorder of cholesterol biosynthesis, NPC disease involves defective cholesterol trafficking associated with an acidic calcium store defect, and Tangier disease is a reverse cholesterol transport disorder. However, a recent serendipitous discovery has highlighted an unanticipated mechanistic link present in all three diseases: the presence of perturbations in the NPC pathway. This discovery is

shedding light on convergent pathogenic mechanisms and suggesting novel approaches to therapeutic interventions in these severe human disorders.

Smith–Lemli–Opitz Syndrome and Niemann–Pick Disease Type C

The first evidence of a potential mechanistic link between SLOS and NPC disease came from studies using SLOS fibroblasts. In principle, SLOS cells should be correctable with cholesterol replacement therapy, as this would bypass the genetic defect in the conversion of 7DHC to cholesterol (**Figure 1**). However, when Wassif et al. (132) cultured SLOS patient fibroblasts in a lipid-depleted medium to induce de novo cholesterol synthesis—which, by the nature of the defect, elevated 7DHC—these cells exhibited a significant cholesterol trafficking defect leading to the accumulation of unesterified cholesterol in the late endosome/lysosome. Sequestration of intracellular cholesterol in the endolysosomal compartment would decrease the bioavailability of cholesterol for other cellular functions in SLOS cells (132), a cellular phenotype that superficially mimics the fate of LDL-derived cholesterol in NPC cells.

The question posed by this study was, did this superficial similarity between these two apparently unrelated disorders suggest a mechanistic convergence? The cholesterol precursor 7DHC, although structurally very similar to cholesterol, has a nonplanar B ring—could (for example) the increased levels of 7DHC be inhibiting NPC1 or NPC2 function, causing exogenous cholesterol to be mistrafficked in SLOS? 7DHC could conceivably interfere with this process by acting as an inhibitor, analogous to U18666A, a drug that induces NPC cellular phenotypes (67). If this hypothesis was correct, then the cell biological and biochemical features of NPC cells should be present in SLOS cells in addition to the SLOS-specific accumulation of 7DHC.

To investigate this possibility, Wassif and colleagues analyzed the cellular and biochemical hallmarks of NPC disease in SLOS patient fibroblasts that spanned the phenotypic spectrum (C. Wassif, E. Lloyd-Evans, L.J. Haslett, I.M. Williams, L. Davis, et al., manuscript in preparation). The results showed that the accumulation of 7DHC in SLOS led to lysosomal storage of cholesterol, sphingomyelin, and multiple GSLs, all of which are hallmarks of NPC disease (**Figure 3**; see also sidebar, Shared Phenotypes of Cholesterol Metabolism Disorders). Furthermore, the SLOS cells had the same lysosomal calcium defect identified as a unique feature of NPC disease, induced by sphingosine storage (69); the study by Wassif and colleagues showed that this calcium defect was responsible for defective transport of cholesterol out of the endocytic system in SLOS cells (C. Wassif, E. Lloyd-Evans, L.J. Haslett, I.M. Williams, L. Davis, et al., manuscript in preparation). These defects are all downstream of 7DHC accumulation, and this combination of

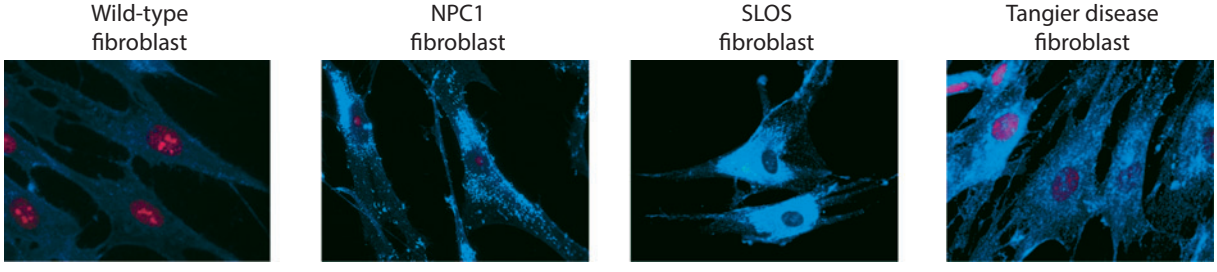

Figure 3

Cholesterol storage (filipin staining) in the late endocytic compartment. This storage is a common feature observed in Niemann–Pick disease type C1 (NPC1 disease), Smith–Lemli–Opitz syndrome (SLOS), and Tangier disease patient fibroblasts relative to a healthy (wild-type) control cell. Filipin is shown in blue, and the nuclear stain is shown in red.

SHARED PHENOTYPES OF CHOLESTEROL METABOLISM DISORDERS

SLOS, NPC disease, and Tangier disease share several key biochemical and cell biological phenotypes:

- Glycosphingolipid storage
- Cholesterol storage
- Sphingomyelin storage
- Sphingosine storage
- Sphingolipid mistrafficking
- Reduced acidic store calcium levels
- Response to miglustat

Miglustat has been approved by the European Medicines Agency for clinical use with NPC; it improves NPC phenotypes in both SLOS and Tangier cells and also improved the symptoms of one patient with Tangier disease.

phenotypes had previously been reported only in NPC disease (69). These data suggest a potential interaction between 7DHC and the NPC1 or NPC2 protein. The same study also showed an inverse correlation between residual enzyme activity (DHCR7) and levels of GSLs and sphingosine storage. Indeed, sphingosine levels in SLOS patient cerebrospinal fluid were elevated 2-fold compared with controls, and this result was replicated in the brain from the full null mouse model at embryonic day 18.5 (C. Wassif, E. Lloyd-Evans, L.J. Haslett, I.M. Williams, L. Davis, et al., manuscript in preparation). GSL levels in the cerebrospinal fluid of SLOS patients were 2.5-fold higher than those of controls, but this did not correlate with severity or residual enzyme activity.

Niemann–Pick Disease Type C and Tangier Disease

Several studies have linked ABCA1 expression/function to the NPC pathway (8, 9). These findings are consistent with the general homeostatic network that exists to regulate cholesterol trafficking and cholesterol levels in cells; a perturbation in one element will have an impact on other pathways in the regulatory network. However, a recent unanticipated finding has suggested a specific mechanistic link between NPC disease and Tangier disease. Interestingly, this link came to light not in the laboratory but from a clinical observation following a diagnostic error. An adult female patient presenting with thrombocytopenia, splenomegaly, macroglossia, dysphagia, ataxic gait, lower-limb lymphedema, and itching nodular skin lesions resembling prurigo nodularis was misdiagnosed with NPC disease and put on the current European Union–approved treatment for NPC, miglustat (93, 137). After six months of treatment with 300 mg/day of miglustat, the patient demonstrated improvement in her neurological symptoms and skin lesions (A. Sechi, A. Dardis, S. Calandra, S. Zampieri, V. Maruotti, et al., manuscript in review). The patient was subsequently diagnosed with Tangier disease after molecular testing of NPC1 and NPC2 failed to support the NPC diagnosis.

Although the neurological symptoms in Tangier disease can be relapsing and remitting, the correlation of symptoms with miglustat therapy was intriguing and suggested a potential mechanistic convergence between NPC disease and Tangier disease, which was then further explored. NPC disease is characterized at the cellular level by storage of GSLs, fatty acids, cholesterol, sphingomyelin, and sphingosine. NPC cells also have low levels of calcium in the late

endosome/lysosome. All of these cellular hallmarks were found in the Tangier disease patient cells (A. Colaco, L. Davis, A. Dardis, N. Al Eisa, D. te Vruchte, et al., manuscript in preparation; see sidebar, Shared Phenotypes of Cholesterol Metabolism Disorders), suggesting that the loss of function of ABCA1 inhibits the NPC pathway through an unknown mechanism. An investigation of *NPC1* and *NPC2* gene expression levels in Tangier disease fibroblasts showed that *NPC2* expression was upregulated twofold, suggesting that this gene's function may be impaired in response to ABCA1 dysfunction (A. Colaco, L. Davis, A. Dardis, N. Al Eisa, D. te Vruchte, et al., manuscript in preparation).

An Expanded Application of Miglustat to Smith–Lemli–Opitz Syndrome and Tangier Disease?

Miglustat is an imino sugar drug that inhibits glucosylceramide synthase, the enzyme that catalyzes the first step in GSL biosynthesis (96, 97). This orally available drug can therefore pharmacologically inhibit the biosynthesis of GSLs to treat lysosomal storage diseases that store GSLs as primary or secondary metabolites (96). Miglustat was approved by the European Medicines Agency in 2002 and by the US Food and Drug Administration in 2003 for the treatment of type 1 Gaucher disease (21, 62). Gaucher disease results from an inherited defect in glucocerebrosidase that leads to the storage of glucosylceramide in the lysosome. Miglustat reduces the number of GSL molecules synthesized by cells so that fewer need to be degraded in the lysosome, allowing the rate of biosynthesis to better match the impaired rate of catabolism (63, 96). Because the drug crosses the blood–brain barrier, it can also potentially treat CNS manifestations of lysosomal storage diseases involving GSL storage (27, 56, 98). Miglustat testing in an NPC1 mouse model demonstrated its efficacy (140), and following an international clinical trial (93), the European Medicines Agency approved it for the treatment of NPC disease. Indeed, it is now approved in most countries, with the exception of the United States (92).

The finding that SLOS and Tangier disease involve secondary inhibition of the NPC pathway (A. Colaco, L. Davis, A. Dardis, N. Al Eisa, D. te Vruchte et al., manuscript in preparation; C. Wassif, E. Lloyd-Evans, L.J. Haslett, I.M. Williams, L. Davis, et al., manuscript in preparation; see sidebar, Shared Phenotypes of Cholesterol Metabolism Disorders) suggests that miglustat could be a potential therapy for these diseases in addition to NPC disease. **Figure 3** shows images of wild-type, NPC1, SLOS, and Tangier disease fibroblasts to illustrate that cholesterol storage in the late endocytic compartment is a common feature of all three diseases. Miglustat treatment of cultured SLOS cells normalizes cholesterol trafficking, with the cholesterol being delivered to the ER. SLOS patients display both fixed developmental abnormalities and functional deficits owing to altered sterol membrane composition. Functional abnormalities in SLOS result from both a cholesterol deficiency and the toxicity of elevated dehydrocholesterol levels. Current therapeutic approaches involve dietary provision of cholesterol and upregulation of DHCR7 activity to increase endogenous cholesterol synthesis. Intracellular endolysosomal sequestration of cholesterol in SLOS will further compound the functional cholesterol deficiency and, by limiting the intracellular bioavailability of cholesterol in the NPC-like cellular phenotype, may limit efficacy of potential therapies. Miglustat treatment of SLOS may therefore correct the NPC phenotypes, allowing cholesterol trafficking to normalize and thus potentially increasing the benefit of cholesterol supplementation. In addition, because miglustat crosses the blood–brain barrier, it has the potential to improve cell biological defects associated with inhibition of the NPC pathway, which may improve CNS function. Tangier disease patient cells treated with miglustat showed biochemical and cell biological correction, suggesting that partial correction of the defective NPC pathway may be of therapeutic benefit, consistent with a case report suggesting that miglustat may have

improved the symptoms of a Tangier patient (A. Sechi, A. Dardis, S. Calandra, S. Zampieri, V. Maruotti, et al., manuscript in review).

CONCLUDING REMARKS

Inborn errors of cholesterol metabolism have provided many fundamental insights into normal cholesterol homeostasis and cell biology over several decades. These disorders have been viewed as discrete diseases with their own unique genetic, biochemical, and cell biological consequences that in turn cause the clinical spectrum of symptoms associated with each disease. What has been surprising is that at least three of these diseases—SLOS, NPC disease, and Tangier disease—share a common pathological hallmark at the cellular level, namely inhibition of the NPC pathway. The precise mechanism that inhibits this pathway in SLOS and Tangier disease remains to be fully elucidated. However, these findings are suggesting novel therapeutic approaches to treating SLOS and Tangier disease using drugs that modify the cell biology of NPC disease, such as miglustat. Miglustat has already been inadvertently tested in one case of Tangier disease, with the results suggesting that it may represent a novel therapeutic approach for this currently untreatable disease. Clinical trials of miglustat in SLOS are currently being planned based on the convergent mechanism of pathogenesis shared with SLOS and NPC. Whether other human diseases also involve NPC pathway dysfunction remains to be determined; this question is also currently under investigation, as it may pave the way for novel approaches to therapy for diseases that currently lack effective treatments using approved NPC disease therapeutics.

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