

Niemann-Pick Type C disease: characterizing lipid levels in patients with variant lysosomal cholesterol storage^S

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Abstract A central feature of Niemann-Pick Type C (NPC) disease is sequestration of cholesterol and glycosphingolipids in lysosomes. A large phenotypic variability, on both a clinical as well as a molecular level, challenges NPC diagnosis. For example, substantial difficulties in identifying or excluding NPC in a patient exist in cases with a “variant” biochemical phenotype, where cholesterol levels in cultured fibroblasts, the primary diagnostic indicator, are only moderately elevated. Here we apply quantitative microscopy as an accurate and objective diagnostic tool to measure cholesterol accumulation at the level of single cells. When employed to characterize cholesterol enrichment in fibroblasts from 20 NPC patients and 11 controls, considerable heterogeneity became evident both within the population of cells cultured from one individual as well as between samples from different probands. An obvious correlation between biochemical phenotype and clinical disease course was not apparent from our dataset. However, plasma levels of HDL-cholesterol (HDL-c) tended to be in the normal range in patients with a “variant” as opposed to a “classic” biochemical phenotype. Attenuated lysosomal cholesterol accumulation in “variant” cells was associated with detectable NPC1 protein and residual capability to upregulate expression of ABCA1 in response to LDL. Taken together, our approach opens perspectives not only to support diagnosis, but also to better characterize mechanisms impacting cholesterol accumulation in NPC patient-derived cells.—Tängemo, C., D. Weber, S. Theiss, E. Mengel, and H. Runz. Niemann-Pick Type C disease: characterizing lipid levels in patients with variant lysosomal cholesterol storage. *J. Lipid Res.* 2011. 52: 813–825.

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Diagnosing the neuro-visceral lysosomal storage disorder Niemann Pick Type C (NPC) offers considerable challenges on a clinical as well as on a laboratory level. Typically,

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the presentation of NPC is uncharacteristic, with mild motor delay, clumsiness, and falls in preschool years, often accompanied by isolated splenomegaly or hepatosplenomegaly. Among the first distinct neurological signs are deficits in vertical and horizontal eye movements, which are subsequently followed by cerebellar ataxia, dysathria, dysphagia, and intellectual deterioration. Such symptoms pave the way to a frequently rapid neurological decline that often results in death before adulthood. However, alternative courses are not uncommon: For example, the disease may present in neonates with severe hepatic failure and a rapidly fatal course. Moreover, NPC is now increasingly diagnosed in teenagers and adult patients who show predominantly psychiatric symptoms and a milder course of neurological disease. Although a first pharmacological agent has recently been approved for therapeutic use, specific causative therapies are not yet available (1–3).

With an estimated frequency of ~1 in 130,000, NPC disease is rare, and in addition to its diverse clinical symptoms, it may easily fail to be recognized by available routine diagnostic tests. Diagnostic security can sometimes be achieved only by genomic sequencing of the two disease-causing genes, *NPC1* and *NPC2*, out of which variants on both alleles of *NPC1* are causative in the vast majority of families. Genotyping, however, is complicated by numerous sequence variants that have been found, particularly in the large *NPC1* gene, among them several frequent polymorphisms and variants for which disease relevance remains unclear (4). Most patients diagnosed with the disease are compound heterozygous for two different variants, making prediction of putative clinical consequences of the genetic findings difficult. Finally, routine sequencing approaches fail to identify mutations in either *NPC1* or

Abbreviations: FCS, fetal calf serum; HDL-c, HDL-cholesterol; HPCD, 2-hydroxypropyl- β -cyclodextrin; LDS, lipoprotein-depleted serum; NPC, Niemann Pick Type C; qPCR, semi-quantitative RT-PCR; TG, triglyceride.

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NPC2 in up to 10% of patients suspected of NPC. Although some of these cases may be explained by rearrangements in deep intronic or noncoding regulatory elements that are difficult to assess (5), the existence of one or more additional disease-causing genes cannot be excluded.

It is therefore generally accepted that for ensuring the diagnosis of NPC in a patient, a stepwise algorithm should be followed (2). Central to the recommended diagnostic guidelines is the establishment of a fibroblast culture from a skin biopsy. To date, in the primary laboratory diagnostic test for NPC, cultured cells are stained with the fluorescent polyene antibiotic filipin, which specifically binds to 3-hydroxysterols such as free cholesterol (6). This allows the experienced diagnostician to evaluate the accumulation of unesterified cholesterol in perinuclear vesicular compartments that correspond to lysosomes (3, 7).

Sequestration of cholesterol and further lipids in lysosomes of neuronal and nonneuronal tissues is a central hallmark of all clinical manifestations of NPC. Both NPC1 and NPC2 proteins have been shown to physically bind free cholesterol deriving from cholesteryl esters that are taken up into the endocytic system from LDL particles. According to a recently established model, it is now believed that within the lysosomal matrix, cholesterol is first bound by NPC2 before being handed over to NPC1, which confers its export from lysosomes (8–10). Whereas in normal cells, LDL-derived cholesterol is rapidly exported from endo-/lysosomal compartments to other organelle membranes, the absence of either NPC1 or NPC2 leads to a massive build-up of cholesterol, glycosphingolipids, and further lipids in lysosomes. However, up to 20% of NPC patients are diagnosed with a “variant” biochemical phenotype that is characterized as a graded, less-severe impairment of intracellular cholesterol trafficking (7). Filipin staining of cultured fibroblasts from such patients is often only mildly elevated when compared with cells from healthy individuals, reflecting a less-severe cholesterol accumulation in lysosomes. Moreover, analyzing cholesteryl ester formation from free cholesterol, a laborious alternative biochemical approach to test for NPC disease, is frequently inconclusive in these patients (3, 7). Although children with neonatal disease onset typically also show the most striking cellular cholesterol abnormalities, the “variant” NPC phenotype is suggested to be overrepresented in patients with neurological signs not before adulthood (7, 11). However, because even the most-severely affected patients may present with only moderate cholesterol accumulation (12), it is generally believed that the extent of cholesterol transport inhibition in fibroblasts does not necessarily correspond to clinical severity.

Thus, the difficulties in identifying or excluding NPC disease in such patients are frequently considerable, which may further protract the diagnostic process and preclude early access to treatment for these patients. Because available therapeutic options are believed to be most efficient at early stages of the disorder (2, 13), tools that may improve early disease detection are urgently sought after.

We have previously analyzed cellular cholesterol levels and distribution in tissue culture cells using the filipin test

on a high-content screening microscopy platform on which microscopic images are acquired automatically and quantitatively analyzed by customized cell recognition software (14, 15). Here, we have established such technology to quantify cholesterol storage in fibroblasts cultured from skin biopsies of individual NPC patients. We show that our approach is well-suited to readily detecting cholesterol accumulation, on both the level of single cells as well as within the population of cells from one patient sample. By applying our approach to patient fibroblast cultures that had been previously diagnosed as showing either “variant” or “classic” (i.e., visually pronounced) cholesterol enrichment, we demonstrate that the biochemical NPC phenotype encompasses a spectrum of diverse cellular phenotypes that show no obvious correlation with disease onset or clinical course. However, other than for patients with marked cholesterol accumulation, plasma levels of HDL-cholesterol (HDL-c) tend to be within the normal range in biochemically “variant” NPC patients. Because our approach not only allows for objective quantification of filipin levels, but also offers high test sensitivity, we introduce quantitative microscopy as an effective tool to support cell-based NPC diagnosis, particularly for patients in which visual interpretation of the NPC cellular phenotype is difficult.

MATERIALS AND METHODS

Patients

DNA, cell cultures, blood samples, and/or clinical data from a total of 55 individuals were analyzed (see supplementary Table I). Out of these, 44 had either previously been diagnosed or were strongly suspected to be affected by NPC disease on the basis of clinical, biochemical, and/or molecular criteria (2). Additionally, cell cultures were analyzed from four obligate heterozygotes (unaffected parents or siblings of NPC patients in whom molecular genetic testing had revealed heterozygosity for a single mutation on only one *NPC1* allele), two healthy control individuals, as well as five patients in which NPC disease had been excluded owing to atypical clinical symptoms and the absence of mutations in the *NPC1* and *NPC2* genes. Data on clinical symptoms and disease course were acquired during individual visits of patients at the Heidelberg-Mainz NPC center as well as by retrospectively analyzing patient files. Plasma lipid profiles of 32 patients were obtained from a certified clinical diagnostic laboratory at Mainz University, compared with age- and sex-specific reference parameters (16), and statistically analyzed using the paired two-tailed Student's *t*-test. Chitotriosidase enzyme activities were determined at the biochemical diagnostic laboratory, Mainz University Children's Hospital, as described previously (17). Genotyping and filipin analyses from cultured fibroblasts were performed at the Institute of Human Genetics, University of Heidelberg, according to established protocols (4, 7). For quantitative determination of the NPC biochemical phenotype, we reanalyzed samples from 31 individuals who, in the years 2007–2010, had been classified by visual microscopic analysis in a diagnostic setting by two independent experts as either “classic,” “variant,” or “unlikely NPC.” All subjects were enrolled either by giving their own signed informed consent or with the permission of their representatives. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in a priori approvals by ethical committees at the Medical Faculties in Heidelberg and Mainz.

Antibodies

Mouse monoclonal antibodies against ABCA1 and β -actin were from Abcam and Sigma, respectively. Rabbit polyclonal antibody against NPC1 was from Novus Biologicals (Littleton, CO).

Cell culture, filipin staining, and biological assays

Primary human fibroblast cultures from skin biopsies of 20 patients with clinical symptoms and/or laboratory findings confirming NPC disease, as well as 11 controls (2 healthy volunteers, 4 unaffected heterozygous carriers, and 5 patients with diseases different from NPC) were analyzed. Unless otherwise indicated, cells were cultured at 37°C, 5% CO₂ in DMEM (1 g/l glucose), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 1% amphotericin B (all from PAA Laboratories Inc.), 10% fetal calf serum (FCS) (Biochrom AG). Filipin stainings of cells cultured on glass coverslips were performed as described (7, 14). Briefly, cells were fixed in 3% paraformaldehyde, stained for 30 min at room temperature with 50 μ g/ml filipin III (Sigma) in PBS from a stock solution of 1 mg/ml in dimethylformamide, and counterstained with nuclear marker DRAQ5 (Biostatus Limited; Leicestershire, UK). Where indicated, fibroblasts were cultured in 5% lipoprotein-depleted serum (LDS, PAN Biotech GmbH) instead of FCS for 48 h before exposure for a further 24 h to 10% FCS/50 μ g/ml LDL that had been isolated from the blood of healthy, normolipidemic volunteers by KBr density gradient ultracentrifugation, as described previously (14). For analyzing the effects of cholesterol depletion on cellular filipin signals (see supplementary Fig. II), cells grown either under control conditions or in the presence of 5% LDS for 48 h were exposed to 1.5% (w/v) 2-hydroxypropyl- β -cyclodextrin (HPCD; Sigma) for 1–8 h prior to filipin analysis.

RNA interference and gene expression analysis

Primary fibroblast cultures at ~80% confluency were transfected for 72 h using LipofectamineTM 2000 (Invitrogen) with either SilencerSelect[®] small interfering RNA (siRNA) targeting human *NPC1* (sense siRNA sequence: 5'-CCAAUUGUGAUAGCAAUUt-3'; Ambion) or nonsilencing control siRNA (14). Total RNA was isolated using the Invisorb[®] Spin Cell RNA Mini Kit (Invitex; Berlin). Semi-quantitative RT-PCR (qPCR) was performed from 1 μ g of isolated RNA as described (14) using qPCR primer pairs for human *NPC1* (5'-GAGAGTGTGGAATTGCAT-ATGG-3', 5'-GAAGAATCCTGGACAGAGTTCC-3') and β -actin (5'-CGCGAGAAGATGACCCAGAT-3', 5'-TCACCGGAGTCCAT-CACGAT-3') (Sigma).

Image acquisition and analysis

Images were acquired as described (14) on an automated screening epifluorescence microscope (Scan^R system; Olympus Soft Imaging Solutions, Münster, Germany) using 10 \times microscope objectives (plan; numerical aperture 0.4; Olympus Europe) and filter sets for Hoechst and Cy5 (Chroma, Inc.). Prior to imaging, samples were stored at room temperature and protected from light for up to several months. For all samples, identical exposure times (200 ms) in the Hoechst channel (reflecting filipin signal) were used. From each sample, a minimum of 10 images per condition were analyzed upon discarding of frames that did not fulfill predefined criteria when visually quality controlled (14). For automated quantitative phenotypic image analysis, we used the customized image analysis software DetecTiff[®] (15) that was parameterized (see supplementary Fig. I) for optimal detection of intracellular perinuclear signals overlapping with the lysosomal marker LAMP1 in cultured human fibroblasts that had been permeabilized with 0.1% Triton-X-100 (Sigma) (not

shown). Numerical image data were analyzed using Bioconductor v 2.5 (<http://bioconductor.org>) under the open-source software package R (v 2.10.0). Algorithms for visual display of acquired data are available upon request. Western blots were quantified using the ImageJ gel analysis tool, and protein levels were normalized to β -actin.

Mutational analysis

Genomic DNA was isolated from EDTA-blood samples. Molecular genetic analyses of the full coding as well as adjacent intronic regions of *NPC1* (NM_000271; exons 1–25) and *NPC2* (NM_006432; exons 1–5) genes were performed as described previously (4, 18, 19), with the adenine in the start codon counting as base #1. DNA sequences were analyzed using Sequence Pilot software v 3.2.1.5 (JSI Medical Systems GmbH; Kippenheim, Germany). Newly identified DNA sequence variants have been entered into the NPC disease gene variation database (4). The following five frequent single-nucleotide polymorphisms at the *NPC1* locus were reported for all individuals: c.387T>C (p.Y129Y; rs12970899), c.644A>G (p.H215R; rs1805081), c.1926C>G (p.M642I; rs1788799), c.2572A>G (p.I858V; rs1805082), and c.2793C>T (p.N931N; rs7229671) (see **Table 1**) (20).

Lipid analyses

Fibroblasts cultured under control conditions were harvested in PBS/0.1% SDS at room temperature and sheared through a 27 G needle; total cellular levels of free cholesterol were determined using the Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer's instructions and analyzed as described previously (14).

RESULTS

Automated image analysis reliably quantifies cholesterol levels in NPC patient fibroblasts

A typical feature of NPC disease is impaired intracellular transport and homeostasis of free cholesterol. In humans, the diagnosis of NPC must be considered when lysosomal cholesterol levels are elevated in a patient's cells. As a sensitive and specific diagnostic assay for NPC, the filipin test is generally well accepted, and relies on staining of fibroblasts cultured from a skin biopsy with the cholesterol binding dye filipin (6, 7). Compared with cells from a healthy control individual in which mutations in the *NPC1* or *NPC2* genes had been excluded (**Fig. 1**; *neg. ctrl 1*), fibroblasts from patients with NPC disease (**Fig. 1**; Δ *NPC1_v03*, Δ *NPCx_c01*) show increased filipin signal intensities in perinuclear compartments (**Fig. 1A**, upper panel). Although this increase is pronounced in patients with a "classic" NPC biochemical phenotype (**Fig. 1A**, right), a considerable fraction of patients show only moderately elevated filipin signals (**Fig. 1A**, middle), implying a less-severe cholesterol storage. This "variant" NPC biochemical phenotype (7) is visually often difficult to distinguish from cells of unaffected individuals, which may contribute to complicating the diagnosis in a patient (2). Therefore, tools are needed that allow for a more objective assessment of cholesterol storage in patient cells.

We have recently established the image analysis software DetecTiff[®] (15). This software allows fully automated object recognition and quantitative analysis of image data

TABLE 1. Biochemical and molecular genetic findings in biochemically “variant” NPC-patient cohort

No	Patient ID	Clinical Diagnosis NPC	Chito-triosidase Activity Elevated ^a	Filipin Phenotype	NPC1 Gene Variants							NPC2 gene variants			
					Mutation Allele 1	Mutation Allele 2	p.Y129Y	p.H215R	p.M642I	p.I858V	p.N931N	Further Variants	Mutation Allele 1	Mutation Allele 2	Further Variants
1.	ANPC1_v01	Yes	Yes	Variant	p.P1007A	p.R934X	+/-	+/-	+/+	+/-	+/-	+/-	n.i.	n.i.	-/-
2.	ANPC1_v02	Yes	Yes	Variant	p.P1007A	p.S940L	-/-	+/+	+/+	+/+	+/+	+/+	n.i.	n.i.	-/-
3.	ANPC1_v03	Yes	Yes	Variant	p.I1061T	p.H1016L	+/-	-/-	+/-	-/-	-/-	-/-	n.i.	n.i.	-/-
4.	ANPC1_v04	Yes	Yes	Variant	p.I1061T	p.N222S	+/-	+/-	+/-	+/-	+/-	+/-	n.i.	n.i.	c.-22a>t
5.	ANPC1_v05	Yes	No ^b	Variant	p.P1007A	c.955+5g>a	+/-	+/-	+/-	+/-	+/-	+/-	n.i.	n.i.	c.1947+8g>c
6.	ANPCx_v06	Yes	No	Variant	n.i.	n.i.	-/-	+/+	+/+	+/+	+/+	+/+	n.i.	n.i.	-/-
7.	ANPC1_v07	Yes	Yes	Variant	p.P1007A	n.i.	+/-	+/+	+/+	+/+	+/+	+/+	n.i.	n.i.	-/-

n.i., none identified.

^aChitotriosidase enzyme activity in serum >100 nmol/h/ml.

^bConfirmed homozygous duplication of the *CHIT1* locus.

Supplemental Material can be found at: <http://www.jlr.org/content/suppl/2011/01/17/jlr.P013524.DC1.html>

with high consistency and has been successfully used to identify modifiers of lysosomal cholesterol levels in HeLa cells (14). To objectify the filipin test and support diagnosis of NPC disease, we have configured this software to quantify perinuclear filipin signal intensities from primary fibroblast cultures (Fig. 1A, lower panel; see supplementary Fig. 1). Fibroblasts from one healthy control individual (*neg. ctrl 1*), one patient diagnosed as showing a “classic” NPC biochemical phenotype ($\Delta NPCx_{c01}$), and two NPC patients with visually only moderately elevated filipin signals ($\Delta NPC1_{v01}$, $\Delta NPC1_{v03}$) were chosen to assess the accuracy of image analysis with this software. From a large number of images per sample that were automatically acquired on a high-throughput microscopy platform (14), we selected 11 representative images for which we compared mean perinuclear filipin signal intensities using either DetecTiff[®] or manual quantification with ImageJ software. As expected, images that were acquired from independent fibroblast cultures of the identical individual showed similar mean perinuclear filipin signal intensities (Fig. 1B). Moreover, for all images analyzed, a high degree of concordance between manual and automated image quantification was obtained ($R^2 = 0.968$). Notably, this was even true for samples between which filipin signals varied considerably, e.g., between control fibroblasts and fibroblasts showing a biochemically “classic” NPC phenotype. Filipin signal intensity in cells has been demonstrated to closely reflect cellular levels of free cholesterol when determined biochemically (14, 21, 22). We could confirm this also for different fibroblast cultures by comparing relative filipin signal intensities as quantified by DetecTiff[®] to total cellular levels of free cholesterol (Fig. 1C). Indeed, our automated image analysis was able to clearly distinguish the selected “variant” NPC fibroblasts showing moderately elevated cholesterol levels ($\Delta NPC1_{v01}$, $\Delta NPC1_{v03}$) from either control cells or cholesterol-loaded fibroblasts showing a “classic” NPC biochemical phenotype ($\Delta NPCx_{c01}$, $\Delta NPC2_{c18}$). Taken together, our results demonstrate that automated analysis of (sub) cellular filipin signals by DetecTiff[®] is able to reliably quantify cholesterol levels in primary human skin fibroblasts, suggesting a potential to support the diagnosis of NPC disease from cultured cells.

Filipin signal intensities in NPC patient fibroblasts vary considerably

We considered automated quantitative analysis of filipin signals from NPC patient fibroblasts with a biochemically “variant” phenotype as of particular interest: First, these cells are visually difficult to distinguish from those of unaffected or heterozygote but healthy individuals (7); and second, the reason that lysosomal cholesterol levels are only moderately increased in these patients is still unclear. Using quantitative image analysis by DetecTiff[®] as a diagnostic tool, we therefore reanalyzed fibroblasts from all patients available to us: *i*) that had been diagnosed between 2007 and 2010 in our laboratory as showing a biochemically “variant” NPC phenotype; *ii*) for which fibroblast cultures were still available to us; and *iii*) in

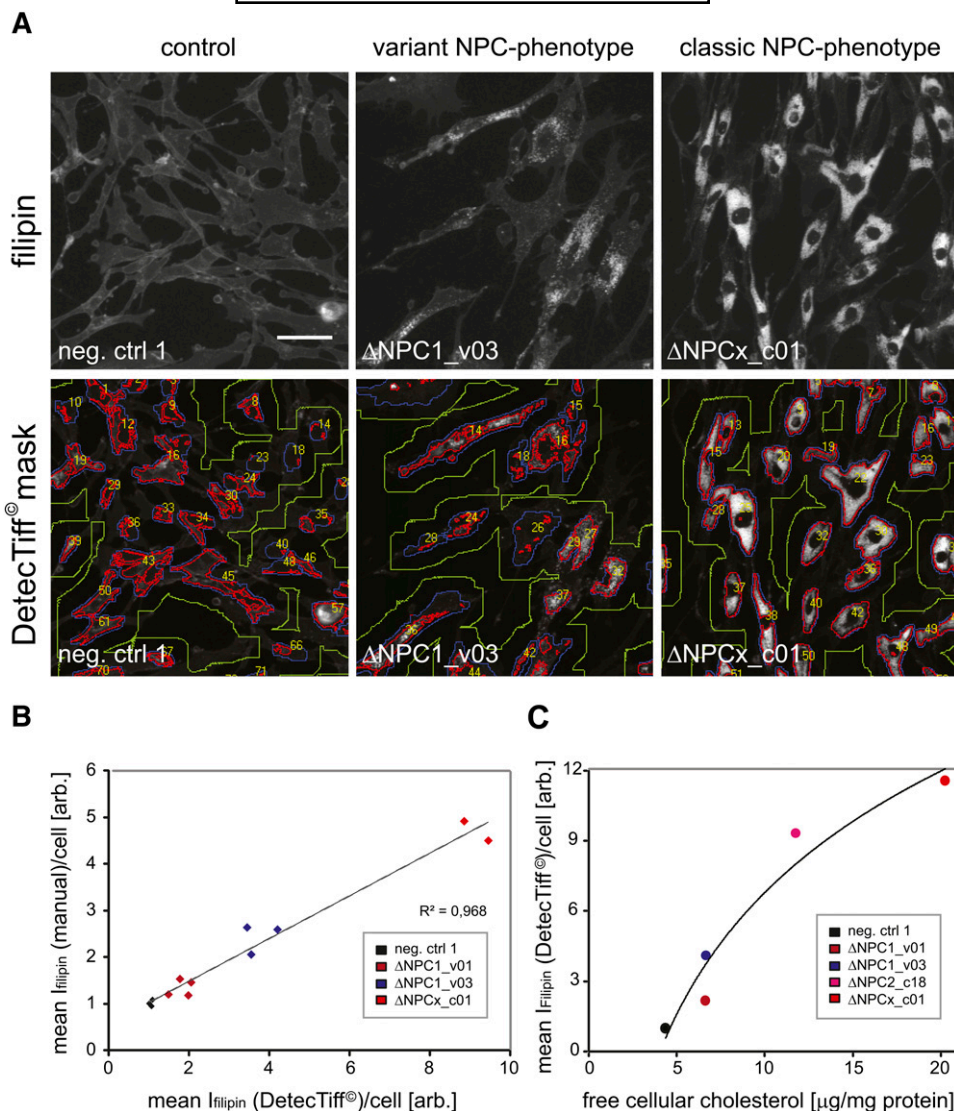


Fig. 1. Automated image analysis reliably quantifies cholesterol levels in Niemann Pick Type C (NPC) patient fibroblasts. (A) Primary human skin fibroblasts from an unaffected individual (*neg. ctrl 1*; left panel), one NPC patient with moderately elevated cellular cholesterol (“variant” biochemical NPC phenotype; $\Delta NPC1_{v03}$; middle panel), and one NPC patient with pronounced cholesterol storage (“classic” NPC-phenotype; $\Delta NPCx_{c01}$; right panel). Cells were cultured under control conditions on glass-bottom slides, fixed, and stained with the cholesterol binding dye filipin. Images were acquired on an automated epifluorescence microscope. Bar = 20 μm . Perinuclear areas encompassing lysosomes (blue/red) were determined from microscopic images with masks generated by the automated image analysis software DetecTiff®. Green lines delineate extra-perinuclear background areas. (B) Scattergraph showing the correlation of mean perinuclear filipin signal intensity/cell within 11 selected images from four different fibroblast cultures, as quantified by DetecTiff® (x axis) relative to manual quantification within masks generated with ImageJ software. On average, 71 cells/image were analyzed. (C) Scattergraph showing the correlation of mean perinuclear filipin signal intensity/cell from five different fibroblast cultures as quantified by DetecTiff® (y axis; means from three to five images/cell line) relative to total cellular levels of free cholesterol as determined biochemically from cell extracts.

which the diagnosis of NPC disease had been confirmed by at least one further disease indicator (2). A total of ten patients fulfilled these criteria. Out of these, detailed clinical as well as comprehensive molecular genetic information could be assessed for seven patients (Table 1). Five of the seven patients tested showed elevated levels of chitotriidase enzyme activity in plasma, a parameter that can be useful for screening for NPC, especially in patients with splenomegaly (2, 17). Six of the patients were compound

heterozygous for at least one *NPC1* mutation that had previously been described as associated with a “variant” NPC biochemical phenotype: p.P1007A (c.3019C>G) (20) and p.I1016T (c.3182T>C) (18), both of which are among the most frequent disease-causing mutations identified in individuals of European ancestry (4). No potentially disease-causing mutation could be identified in the remaining patient ($\Delta NPCx_{v06}$), despite full genomic sequencing of the *NPC1* and *NPC2* coding regions.

We then compared filipin signal intensities as quantified by DetectTiff[®] of the ten “variant” NPC patients to those of ten patients who had been diagnosed as showing a “classic” NPC biochemical phenotype. Moreover, we analyzed cells from four unaffected obligate heterozygote individuals, two healthy controls, and five patients in which NPC disease had been excluded because of atypical clinical signs and negative genotyping of the *NPC1* and *NPC2* genes. Ten out of ten fibroblast cultures previously classified as “classic NPC” showed median filipin signal intensities well above those of heterozygotes, healthy controls, or patients with diseases other than NPC (Fig. 2A). This indicates a high sensitivity of our analysis strategy to identify NPC patients with a “classic” biochemical phenotype. Remarkably, however, the pattern of cellular cholesterol accumulation as indicated by filipin varied considerably, *i*) among the population of cells from one individual patient, and *ii*) between different individuals within each of the five subgroups analyzed. Within the population of fibroblasts cultured from the skin biopsy of patient $\Delta NPC2_c18$, for example, some cells revealed an almost 10-fold stronger filipin signal than did others. Moreover, median filipin signal intensities differed by more than 4-fold when cells from different patients visually classified as “classic NPC”

were compared. Considerable heterogeneity was also detected among the patients previously diagnosed with a “variant” NPC biochemical phenotype: Median filipin signals in two ($\Delta NPC1_v06$; $\Delta NPC1_v07$) of the ten “variant” cell lines overlapped with those observed in “classic” cells. Likewise, median signal intensities in three ($\Delta NPC1_v01$; $\Delta NPC1_v02$; $\Delta NPC1_v10$) of the “variant” fibroblast cultures from patients diagnosed with NPC disease overlapped with those of heterozygotes or patients in which NPC was considered unlikely. Patient $\Delta NPC1_v01$, who had been diagnosed as showing “moderate if at all increased filipin signals,” revealed even lower filipin signal intensities than one of the two healthy reference individuals tested (Fig. 2A).

We confirmed these data by independent visual microscopic reanalysis of all “variant” NPC samples tested (Fig. 2B). With the exception of patient $\Delta NPC1_v07$, who would have now been classified as showing a “classic” NPC biochemical phenotype, samples from all other nine patients were again evaluated as “variant NPC.” In summary, the findings of our quantitative analysis corroborate previous assumptions that the filipin test may be inconclusive in the lower signal intensity range (2, 3) and indicate that in fibroblasts of certain NPC patients, lysosomal cholesterol

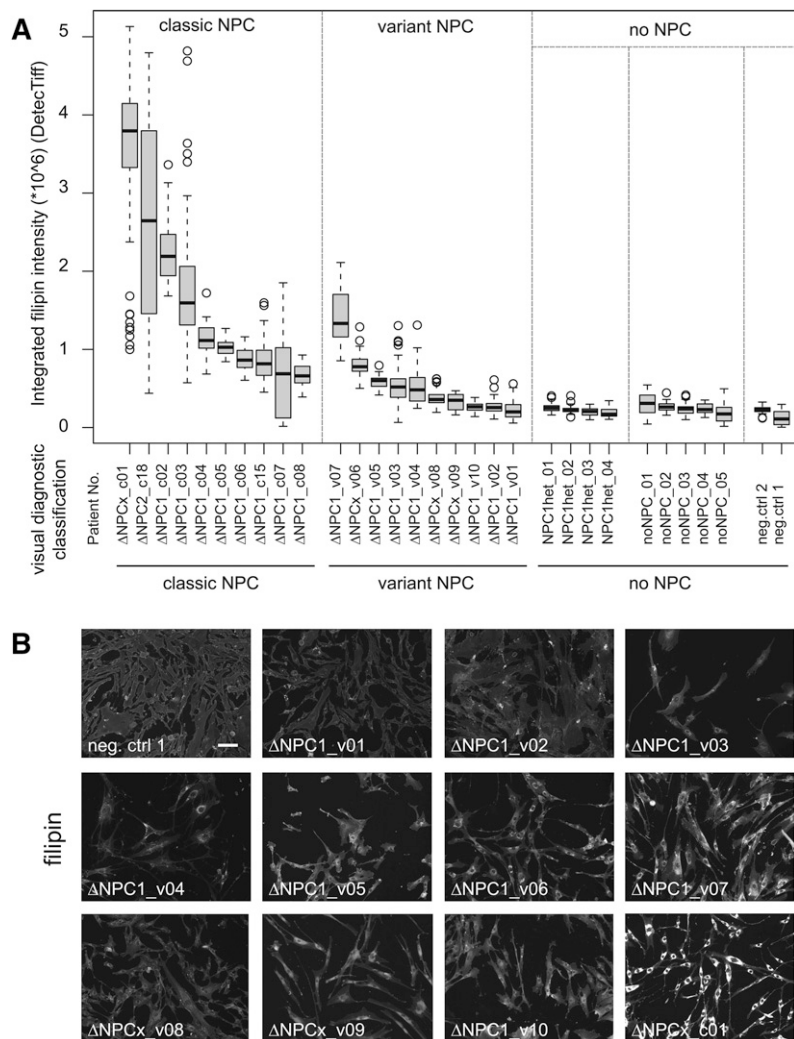


Fig. 2. Analysis of fibroblasts from 20 different NPC patients reveals a continuous spectrum of filipin signal intensities. (A) Comparison of the range of filipin signal intensity distributions (y axis) among fibroblasts from ten NPC patients visually classified as “classic” biochemical phenotype (ΔNPC_c), ten patients classified as “variant” phenotype (ΔNPC_v), four obligate heterozygotes ($\Delta NPC1_het$), two healthy control individuals (*neg. ctrl*), and five patients in which NPC disease had been considered unlikely (*noNPC*) (x axis). Signal intensities were quantified by DetectTiff[®] from, on average, 39 images/cell line (range: 10–144) with data from up to 2,208 fibroblasts/patient. Box plots show medians (bars), lower and upper quartiles (boxes), 10th and 90th percentiles (whiskers), and outliers (○). (B) Representative images from 10 “variant” patients (ΔNPC_v01-10), 1 “classic” patient ($\Delta NPCx_c01$), and 1 healthy control individual (*neg. ctrl 1*). Cells cultured under control conditions on glass coverslips were fixed and stained with filipin, and images were acquired on an automated epifluorescence microscope. Bar = 20 μ m.

levels are not increased beyond those of controls. Moreover, because a clear separation between “classic” and “variant” NPC patients, as well as between “variant” patients and controls, was not evident from our dataset, our data suggest that rather than clustering into distinct uniform subgroups, the biochemical phenotype in NPC patients follows a continuous spectrum.

The NPC biochemical phenotype in patient fibroblasts can be manipulated with cholesterol-modifying agents

Cultivating fibroblasts under specific cell culture conditions can improve the diagnostic value of the filipin test and thus facilitate identification of NPC patients with the “variant” biochemical phenotype (2). When patient fibroblasts cultured under low-cholesterol conditions are loaded with LDL, lysosomal cholesterol storage may become more pronounced (23–25). This is well in line with the overall increase in perinuclear filipin signal intensities that we could detect in fibroblasts cultured according to this protocol and studied with our analysis strategy (Fig. 3). Notably, however, the extent of this increase differed between the different cell lines tested. Filipin signals in fibroblasts from a healthy volunteer (*neg. ctrl 1*) increased only slightly upon exposure to LDL, reflecting an unperturbed cholesterol homeostatic response in these cells. Consistent with the literature, LDL-induced increase in mean perinuclear filipin signals was more pronounced in fibroblasts with a “variant” biochemical phenotype ($\Delta NPC1_{v01}$; $\Delta NPC1_{v03}$), as indicated by a shift of the curves shown in Fig. 3 to the right. Conversely, LDL did not further increase mean filipin signals in NPC fibroblasts with a “classic” biochemical phenotype ($\Delta NPCx_{c01}$; $\Delta NPC2_{c18}$), which suggests that cellular capacity for further storage of cholesterol is saturated in the majority of “classic” NPC cells.

Similar to treatment with LDL, our software was also able to detect increased lysosomal cholesterol levels in lysosomes of control fibroblasts treated with siRNAs against NPC1 (see supplementary Fig. IIA). Conversely, lower filipin signals reflecting a reduction in lysosomal cholesterol storage were detectable upon exposure of “variant” NPC fibroblasts to HPCD (see supplementary Fig. IIB), which has been shown to efficiently reduce the NPC storage phenotype both in cells as well as in NPC mouse models (26–28). Taken together, manipulation of filipin signal intensities in cultured fibroblasts by strategies that have previously been described to increase or reduce lysosomal cholesterol storage is readily detectable with our analysis strategy.

The “variant” biochemical NPC phenotype correlates with normalization of plasma HDL-c levels and increased expression of ABCA1

Why fibroblasts from some patients with NPC disease store less cholesterol than others, and how and if this affects disease pathogenesis and clinical course, are still unclear. We therefore were interested in whether the differences in cellular cholesterol enrichment, as revealed by our semi-quantitative approach, correlate with clinical data. Of the seven patients with a “variant” storage pattern

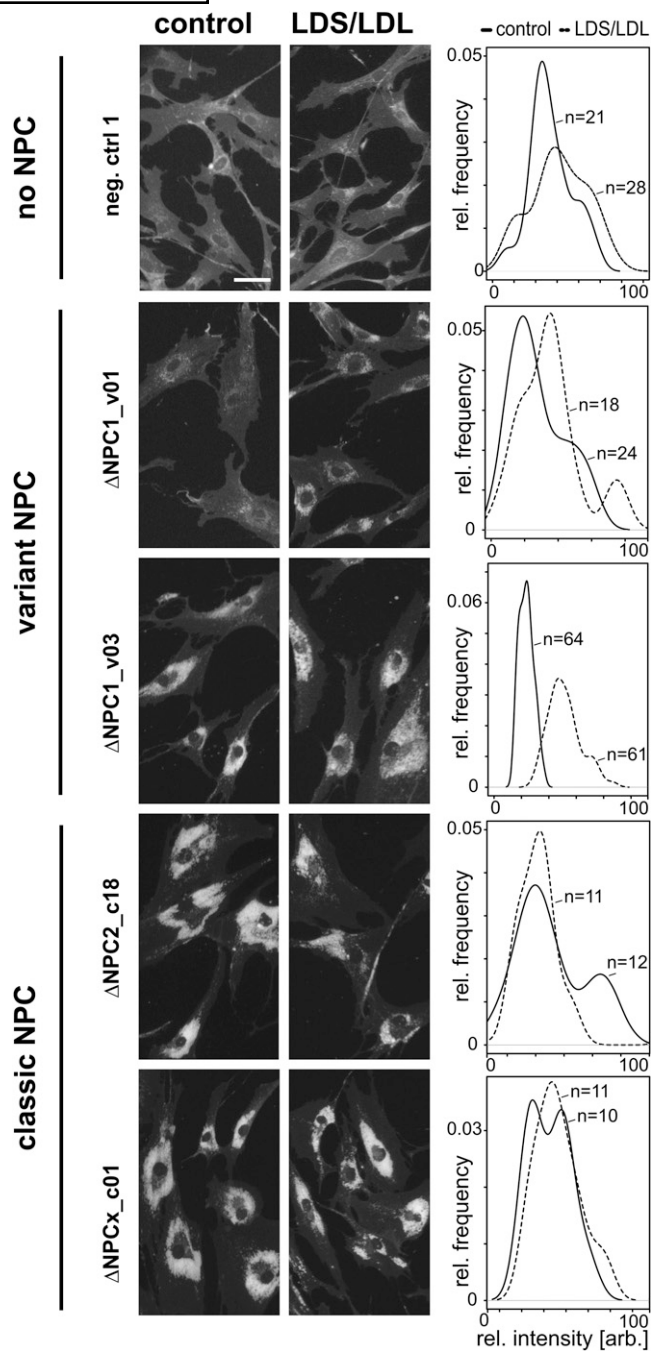


Fig. 3. Filipin signal intensities in “variant” NPC patient fibroblasts may increase upon exposure to LDL. Fibroblasts from one control individual (*neg. ctrl 1*), two NPC patients with a “variant” ($\Delta NPC1_{v01}$; $\Delta NPC1_{v03}$), and two patients with a “classic” biochemical phenotype ($\Delta NPC2_{c18}$; $\Delta NPCx_{c01}$) were cultured either under control culture conditions (10% fetal calf serum; left column) or in lipoprotein-depleted serum (5% LDS) for 48 h before exposure to 50 $\mu\text{g/ml}$ LDL-cholesterol (LDL-c) in 10% FCS for 16 h (middle column). Then cells were fixed and stained with filipin, and images were acquired automatically. Bar = 20 μm . Relative intensity frequency distributions [0..1] (y axis) of mean perinuclear filipin signal intensities as quantified by DetecTiff[®] (x axis) from the indicated number of images/cell line (n), and two to three independent replica experiments were quantified. For better comparison, for each cell line, lowest signals were set to 0%, brightest signals to 100%.

in fibroblasts, four showed first disease symptoms in infancy (<5 years of age), two as juveniles (6–15 years), and one as an adult (35 years) (Table 2). With an average 4.63 years (median: 3.5 years) the diagnostic delay from onset of first disease symptoms to the diagnosis of NPC did not significantly differ from other NPC patients at our NPC clinical center (H. Runz, E. Mengel, unpublished observations) or the literature (29). At the time of the last visit, six of the seven patients with a biochemically “variant” NPC phenotype presented with visceral organomegaly, six patients showed vertical supranuclear gaze palsy, and all patients showed ataxia. Retrospective analysis of clinical data available on these patients did not show evidence for a milder disease course, neither in “variant” cases with infantile disease onset, nor in those with juvenile or adult disease onset (Fig. 4). In summary, although a small sample size, these data do not indicate major differences with regard to disease progression in NPC patients with a “variant” biochemical phenotype, and corroborate previous observations that conclusions from cholesterol storage in fibroblasts as a predictive measure for the clinical course in a patient should be made with care (3).

We were further interested in whether a “variant” cholesterol storage pattern in fibroblasts correlated with systemic changes in blood lipid levels in the respective patients. For this, we analyzed routine laboratory parameters in a cohort of 32 NPC patients for which retrospective laboratory diagnostic data from regular follow-up visits at our NPC center were available (Fig. 5). In addition to the seven “variant” NPC patients, nine of these patients were reported as showing a “classic” biochemical NPC phenotype; for the remaining 16 patients, a distinction between biochemically “classic” or “variant” had not been documented. Compared with the age- and sex-matched unaffected reference population (16), NPC patients of our cohort were overrepresented in the lower 5th percentile group with regard to HDL-c, LDL-c, and free cholesterol, as well as above the 95th percentile with regard to triacylglycerides (TGs). This corroborates previous findings that NPC patients have a higher probability for low HDL-c, LDL-c, and blood cholesterol levels, whereas TG levels tend to be increased (30). No evident differences between biochemically “variant” and “classic” NPC patients were found for LDL-c, cholesterol, and TG. For HDL-c, however, the “variant” patients of our cohort showed significantly higher levels than did the patients with a biochemically “classic” NPC phenotype ($P < 0.01$). In fact, among the “variant” patients, HDL-c levels below the respective lower reference levels were observed only for patient $\Delta NPC1_v04$ and are likely to be secondary, inasmuch as this adult-onset patient also showed highly elevated TG (412 mg/dl) due to metabolic syndrome (BMI 37).

Low HDL-c levels in NPC patients have been explained by an impaired formation of HDL particles owing to corrupted upregulation of ABCA1, when NPC cells are exposed to LDL (31). We therefore hypothesized that one explanation for the relative increase of HDL-c in “variant” compared with “classic” NPC patients could be sufficient ability of these patients to maintain ABCA1 regulation,

TABLE 2. Clinical course, disease symptoms, and blood lipid levels of biochemically “variant” NPC-patient cohort

No	Patient ID	Sex	Disease Class ^a	NPC Disease Course		NPC Disease Clinical Symptoms												Blood Lipid Levels ^b									
				age at first symptoms	age at 1st neurop. symptoms	Age at Diagnosis NPC	Age at Last Visit	Neonatal Liver Disease	Psychomotor Delay	Splenomegaly	Hepato-/ Splenomegaly	Vertical Gaze Palsy	Ataxia	Dysphagia	Cataplexia	Seizures	Pulmonary Disease	HDL-c	LDL-c	Chol	TG						
1.	$\Delta NPC1_v01$	Male	Infantile	0	2	7	12	y ^d	y	y	y	y	y	n	n	n	n	n	n	n	n	n	n	50	46	112	83
2.	$\Delta NPC1_v02$	Female	Infantile	3	3	5	12	n	n	y	y	y	y	n	n	n	n	n	n	n	n	n	n	41	100	165	126
3.	$\Delta NPC1_v03$	Female	Infantile	4	4	5	9	n	n	y ^d	y	y	y	n	n	n	n	n	n	n	n	n	n	41	220+	282+	105
4.	$\Delta NPC1_v04$	Male	Adult	35	44	40	45	n	n	y ^d	y	y	y	n	n	n	n	n	n	n	n	n	n	27-	53	137-	420+
5.	$\Delta NPC1_v05$	Male	Infantile	4	4	10	12	n	n	n	y	y	y	n	n	n	n	n	n	n	n	n	n	39	75	146	160+
6.	$\Delta NPCx_v06$	Female	Juvenile	10	10	12	13	n	n	n	n	y	y	y	y	y	y	y	y	y	y	n	n	41	131	196	117
7.	$\Delta NPC1_v07$	Female	Juvenile	2	13	15	16	n	y ^d	y	y	y	y	y	y	y	y	y	y	y	y	n	n	46	80	147	107

n.v., Symptom present at last visit.

^aDisease class (according to Wraith et al., 2009): early/late infantile (0–6 years), juvenile (6–15 years), adult (> 15 years).

^bMean blood lipid parameters from 1–10 independent measurements.

^cSymptom present at diagnosis.

^dPresenting symptom.

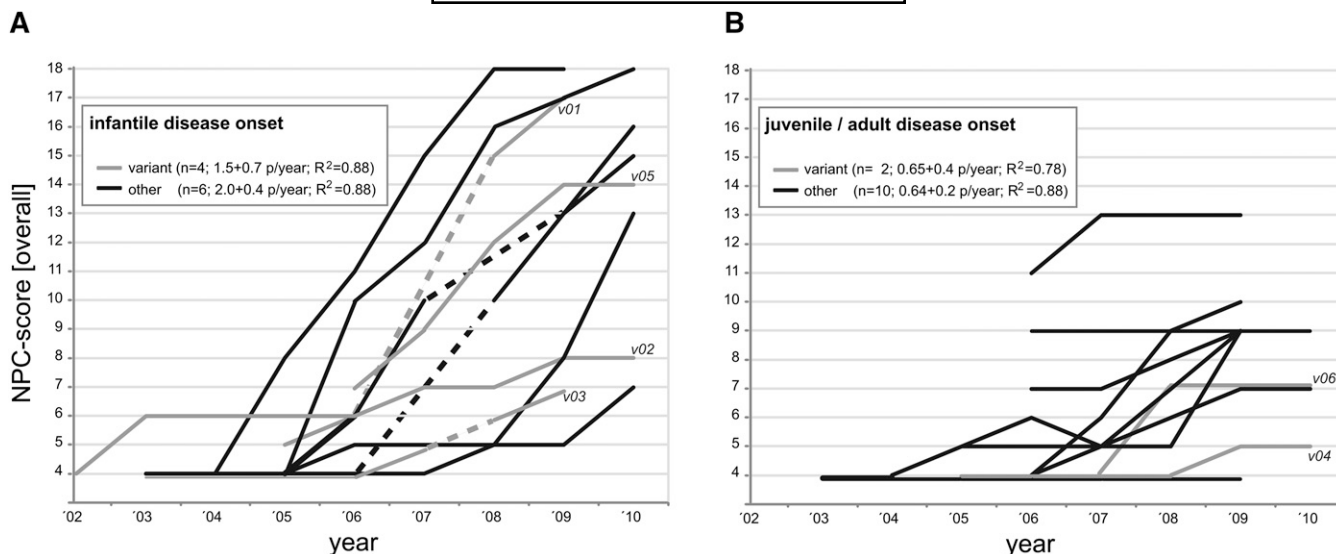


Fig. 4. Patients with a biochemically “variant” phenotype show a disease progression similar to that of other NPC patients. Longitudinal severity scores according to (43) in a cohort of 10 patients with infantile disease onset (<5 years) (A) and 12 patients with juvenile/adult disease onset (>6 years) (B). Scores were assessed by retrospective analysis of clinical data acquired between 2002 and 2010. The progression slope per subgroup (boxes) was determined by linear regression of the depicted curves and averaging for either biochemically “variant” (gray curves) or “other” NPC-patients [i.e., either “classic” patients (n = 6) or patients for which biochemical phenotype was unavailable (n = 10); black curves] separately.

e.g., due to residual activity of NPC1 protein in lysosomes. To test for this, we assessed NPC1 and ABCA1 protein levels in control (*neg. ctrl 1*), “variant NPC” ($\Delta NPC1_{v03}$; $\Delta NPC1_{v05}$), and “classic NPC” ($\Delta NPC1_{c03}$; $\Delta NPC2_{c18}$) fibroblasts cultured under control conditions, or in the absence of sterols, or loaded for 24 h with LDL (Fig. 6). In-

dependent of culture conditions, no NPC1 protein could be detected in the “classic” patient, $\Delta NPC1_{c03}$, whereas, consistent with the literature (19), NPC1 levels were increased in fibroblasts with a “classic” storage pattern owing to deficiency for NPC2 (Fig. 6A). Both “variant” NPC1 mutant cell lines analyzed still expressed NPC1, although at

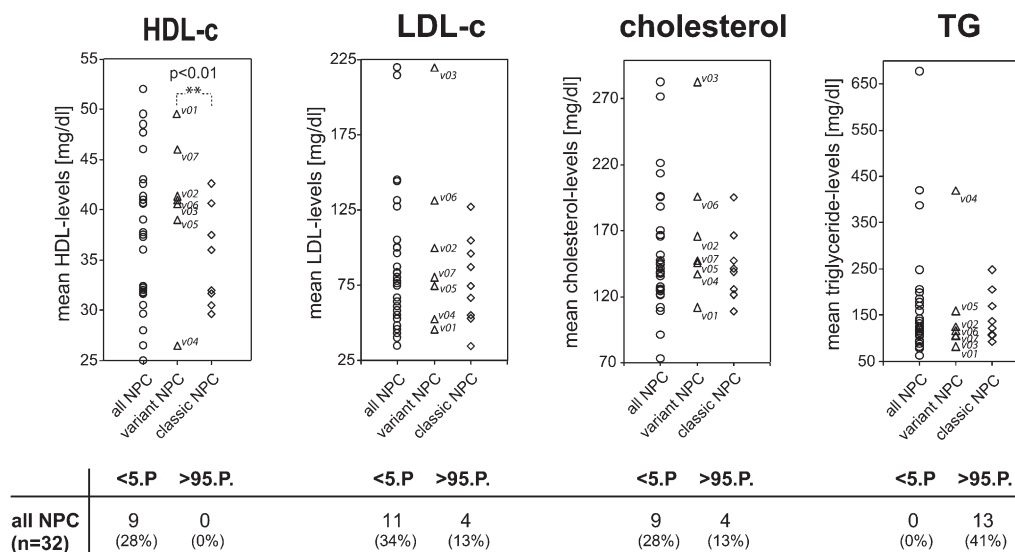


Fig. 5. The “variant” biochemical NPC phenotype correlates with normalization of plasma HDL levels. Mean plasma levels of HDL-c, LDL-c, total cholesterol, and triacylglycerides (TGs) were determined in 32 NPC patients (“all NPC,” left columns, circles), out of which 7 patients had been diagnosed as “variant” (middle columns; triangles) and 9 as “classic” biochemical phenotype (right columns, squares). Each data point represents mean parameters from 1–14 independent blood samples per patient (with on average approximately three independent measurements/patient). Statistical analysis of source data was performed using the paired 2-tailed Student’s *t*-test (** *P* < 0.01). The table shows number of patients/parameter in which mean levels fell below the 5th percentile (<5.P) or above the 95th percentile (>95.P) of the age- and sex-adjusted reference population.

considerably lower levels than controls. As expected for healthy control cells, ABCA1 levels were reduced upon sterol depletion, but upregulated when LDL was loaded to sterol-depleted cells. In agreement with a previous report (31), NPC cells with a “classic” biochemical phenotype were impaired in upregulating ABCA1 when exposed to LDL. Importantly, this impairment was considerably less-pronounced in fibroblasts of the two “variant” patients tested, who, upon loading with LDL, were still able to partially upregulate ABCA1 levels, indicating residual regulatory capability (Fig. 6A, B). Taken together, our data suggest that higher HDL-c levels in biochemically “variant” compared with “classic” NPC patients may be explained by higher levels of residual NPC1 protein in patient cells and sufficient ability to induce the expression of ABCA1 upon challenge with LDL.

DISCUSSION

With its ability to unravel deficits in intracellular cholesterol trafficking and homeostasis, the filipin test is considered the primary diagnostic tool for determining NPC disease in a patient. However, despite high test sensitivity

and specificity, the general applicability of this test is constrained for two reasons: first, it relies on cultured fibroblasts, which necessitates a skin biopsy; and second, cholesterol accumulation between the cells within one sample, but also between samples from different individuals, can be variable, so that an adequate interpretation of the test requires considerable diagnostic experience.

Here we have applied automated microscopy and quantitative image analysis to objectively assess filipin signal intensity and distribution in a large number of fibroblasts from patients with NPC disease, as well as unaffected controls. We show that with this strategy, it is possible to reliably quantify the NPC biochemical phenotype with high accuracy at the level of single cells, and that filipin signal intensities correlate well with cellular levels of free cholesterol. Automated image acquisition offers the advantage of constant autofocus and illumination times, which are particularly helpful when imaging rapidly bleaching dyes such as filipin (14, 15). Moreover, it allows extraction of reliable quantitative information from many cells with little effort and usually low statistical noise, even in cell types with a profound heterogeneity in cell growth and morphology, such as primary fibroblast cultures from different individuals. Although it may still fall short when compared with visual interpretation by an experienced diagnostician, we are confident that automation may be a substantial aid in filipin-based NPC diagnostics, especially in cases in which diagnosing NPC by visual means is difficult.

Although the filipin test is highly reliable in the majority of NPC patients, it is generally accepted that in certain individuals, an adequate diagnosis is particularly challenging. For example, specificity of the test is limited in fibroblasts from patients with mucopolipidosis type II, because these cells may also retain massive amounts of cholesterol and may be indistinguishable from those of NPC patients with a biochemically “classic” phenotype (7). Likewise, fibroblasts from NPC heterozygotes and other disorders, such as NP-A/B or Wolman disease, may reveal mildly abnormal filipin patterns and thus can be mistaken as showing a biochemically “variant” NPC phenotype (2, 3, 7, 25). Corresponding to visual analysis, our diagnostic strategy proved highly sensitive in identifying fibroblast cultures previously classified as “classic NPC”; however, like visual analysis, it was limited to clearly distinguish cells reported as “variant NPC” from both, “classic” patients as well as unaffected controls. Based on our unbiased retrospective analysis of samples from many different individuals with and without NPC disease, we believe that the most likely explanation for this is that cholesterol enrichment in healthy and diseased fibroblasts as reflected by filipin follows a continuous spectrum rather than defining distinct uniform subgroups. Therefore, to determine or exclude NPC disease in a patient with only a little cholesterol storage, further diagnostic tools such as genotyping should be considered essential (3).

One strategy to partially overcome the diagnostic predicaments of the filipin test is to expose cholesterol-depleted cells to LDL, which, owing to corrupted cholesterol homeostatic responses in NPC cells, may further increase

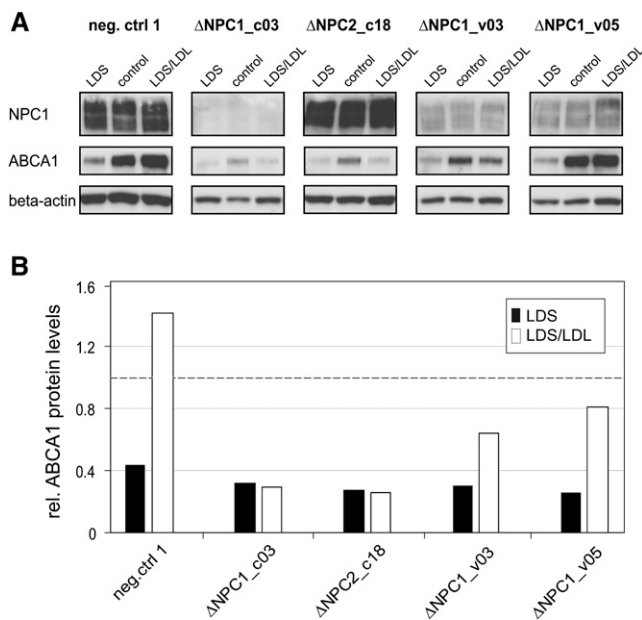



Fig. 6. “Variant” NPC fibroblasts show residual NPC1 protein expression and LDL-dependent upregulation of ABCA1. (A) Lysates from fibroblasts of one healthy control individual (*neg. ctrl 1*), two NPC patients with “classic” ($\Delta NPC1_{c03}$; $\Delta NPC2_{c18}$), and two patients with “variant” biochemical phenotype ($\Delta NPC1_{v03}$; $\Delta NPC1_{v05}$) were subjected to Western blot and analyzed with antibodies against NPC1, ABCA1, or β -actin, respectively. Cells were cultured either under control conditions (DMEM/10% FCS; middle lane), depleted from sterols by culture in 5% LDS instead of FCS for 72 h (LDS; left lane), or upon sterol depletion for 48 h challenged with 10% FCS/50 μ g/ml LDL for 24 h (LDS/LDL; right lane). (B) ABCA1 protein levels under control, sterol-depleted (LDS), and LDL-exposed (LDS/LDL) conditions in (A) were quantified and normalized to β -actin on the identical lane. For each respective patient, relative ABCA1 levels under the indicated culture condition were normalized to control conditions (= 1).

cholesterol accumulation (32, 33). Consistent with the literature, such response in “variant” cell lines could indeed be observed also with the semi-quantitative approach presented here. However, loading of LDL-c either to control cells with intact cholesterol homeostasis or to NPC fibroblasts with a “classic” storage pattern further increased filipin signal intensities only a little, if at all. One possible explanation for this could be the broad distribution of filipin signal intensities we observed, particularly in cultures from “classic” NPC patients: Within the same sample, cells with a high storage capacity for cholesterol coexisted with cells that seemed to be already saturated at lower amounts of storage material but in which cholesterol homeostatic responses nevertheless were likely to be maximally corrupted. The possibility that NPC fibroblast cultures are mosaics of cells that respond to an environmental stimulus such as LDL or also cyclodextrins, to a different extent may be of relevance for large-scale screening approaches that apply the filipin test to query for substances that may alleviate cholesterol storage in NPC disease (34, 35). Indeed, assessing the distribution of filipin signal intensities within a population of individual cells rather than averaging parameters over the whole population proved a successful strategy in a recent filipin-based siRNA screen in NPC cells (36). “Variant” NPC cells may be an ideal phenotypic read-out for such approaches, because accumulated cholesterol is likely to be mobilized more easily and secondary effects induced by lysosomal dysfunction may not yet be so severe.

Much discussion has revolved around the issue of whether cholesterol is the primary storage product in the disease process, or if its accumulation occurs rather secondarily to that of other lipids (37, 38). Similarly, the correlation between cholesterol accumulation in fibroblasts and either NPC genotype or clinical course, respectively, has not yet been fully clarified. The findings in our patient cohort support the assumption that the extent of cholesterol transport inhibition in fibroblasts is of only limited predictive value for conclusions on clinical severity. For example, $\Delta NPC1_{v01}$, our patient with filipin signal intensities overlapping with those of controls, presenting with liver disease as a neonate, showed delayed motor milestones in early infancy and died at the age of 12 years shortly before submission of this manuscript. Also, pronounced differences in disease progression between patients classified as biochemically “variant” and others were not obvious from our cohort. Our data, however, did support hints at a putative relationship between the extent of cellular cholesterol accumulation and the *NPC1* genotype. Four of the seven “variant” patients were heterozygous for the frequent *NPC1* mutation p.P1007A (c.3019C>G), which has been described as sufficient to maintain residual cholesterol export from lysosomes (18, 19). Although homozygosity for the mutation p.I1061T is known to induce a “classic” NPC phenotype, cholesterol storage may be alleviated when this variant, as found in two of our patients, cooccurs in trans with a mutation that introduces an amino acid substitution in the cystein-rich loop domain of the *NPC1* protein (11, 18). It is noteworthy that all mutations

except the newly identified *NPC1* variant p.N222S (c.665A>G) localize to this luminal loop domain, which is believed to be important for interaction with yet-unidentified binding partners (37). Despite full genomic sequencing of both disease-associated genes, we were unable to find causative variants in patient $\Delta NPCx_{v06}$. Although this may not be due to shortcomings of routine genomic sequencing in identifying deep intronic *NPC1* mutations (5), one previous study reported a high coincidence of the “variant” cellular phenotype with no apparent defects in the *NPC1* gene (39). Therefore, it is tempting to speculate that variants in additional, yet-unidentified genes have an impact on the NPC cellular phenotype, and modifiers of lysosomal cholesterol levels have been demonstrated in cell and animal models (e.g., 14, 30, 40).

A role for additional genes in the phenotypic expression of NPC disease on a cellular level is further supported by the results of plasma lipid analyses in the patients presented here. Most interestingly, not only patient $\Delta NPC1_{v03}$, but also one affected sibling and several unaffected relatives of this patient showed highly elevated plasma levels of LDL-c and free cholesterol, indicative of familial hypercholesterolemia in addition to NPC disease in that family (H. Runz, unpublished observations). By comparing mean lipid parameters in our cohort of NPC patients to age- and sex-matched individuals of the unaffected reference population (16), we could further confirm the findings of one previous study (30) that NPC patients show a tendency toward low HDL-c, LDL-c, and blood cholesterol, but high TG levels. It was also reported previously that the reduction in HDL-c tended to be stronger in patients with a more severe impairment of intracellular cholesterol trafficking (30, 31). This is well in line with our finding reported here that unlike patients with a “classic” biochemical phenotype, NPC patients with a “variant” storage pattern tend to show HDL-c levels within the normal range. Although the intracellular distribution of cholesterol delivered from HDL particles, as well as the cellular export of endogenously synthesized or even excessively stored cholesterol, does not seem to depend on functional *NPC1* protein (41, 42), *NPC1*-deficient cells are impaired in cholesterol-dependent activation of ABCA1 (31), the rate-limiting protein in the formation of HDL particles. Our current data propose that this impairment is less pronounced in NPC fibroblasts with a “variant” as compared with a “classic” biochemical phenotype and correlates with residual levels of *NPC1* protein. To elucidate: whether the residual capability of “variant” NPC cells to induce ABCA1 expression in response to LDL is secondary to a less-severe cholesterol transport block from lysosomes, or rather could hint at yet-unknown pathways amenable to therapeutic interventions that restore cellular cholesterol homeostasis in NPC cells will need to be elucidated in future studies. 

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