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Ontogenic changes in lung cholesterol metabolism, lipid content, and histology in mice with Niemann-Pick type C disease

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Abstract

Niemann-Pick Type C (NPC) disease is caused by a deficiency of either NPC1 or NPC2. Loss of function of either protein results in the progressive accumulation of unesterified cholesterol in every tissue leading to cell death and organ damage. Most literature on NPC disease focuses on neurological and liver manifestations. Pulmonary dysfunction is less well described. The present studies investigated how *Npc1* deficiency impacts the absolute weight, lipid composition and histology of the lungs of *Npc1*^{-/-} mice (*Npc1*^{nih}) at different stages of the disease, and also quantitated changes in the rates of cholesterol and fatty acid synthesis in the lung over this same time span (8 to 70 days of age). Similar measurements were made in *Npc2*^{-/-} mice at 70 days. All mice were of the BALB/c strain and were fed a basal rodent chow diet. Well before weaning, the lung weight, cholesterol and phospholipid (PL) content, and cholesterol synthesis rate were all elevated in the *Npc1*^{-/-} mice and remained so at 70 days of age. In contrast, lung triacylglycerol content was reduced while there was no change in lung fatty acid synthesis. Despite the elevated PL content, the composition of PL in the lungs of the *Npc1*^{-/-} mice was unchanged. H&E staining revealed an age-related increase in the presence of lipid-laden macrophages in the alveoli of the lungs of the *Npc1*^{-/-} mice starting as early as 28 days. Similar metabolic and histologic changes were evident in the lungs of the *Npc2*^{-/-} mice. Together these findings demonstrate an intrinsic lung pathology in NPC disease that is of early onset and worsens over time.

Keywords

pulmonary; alveoli; phospholipid; triacylglycerol; liver

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1. Introduction

In Niemann-Pick type C (NPC) disease unesterified cholesterol (UC), gangliosides, and glycosphingolipids continually accumulate in the late endosomal/lysosomal (E/L) compartment of all cells in every organ. It is an autosomal recessive disorder primarily affecting children and is characterized by neurodegeneration, hepatic and pulmonary dysfunction, and premature death [1]. A lot of what we currently know about NPC1 and NPC2 function, and also the etiology and pathogenesis of NPC disease has come from the study of animal models with NPC1 or NPC2 deficiency, primarily in the mouse [2–14], and also from various in vitro systems as well as techniques such as X-ray crystallography [15–17]. Unequivocally, it is the sequestration of UC in the E/L compartment that is the fundamental cause of NPC disease [18]. This accumulation of UC is due to a loss of function mutation in genes encoding either NPC1 or NPC2, both of which are essential for facilitating cholesterol transport out of the late E/L compartment [17]. Disruption of this egress and the resultant buildup of UC, together with that of other types of lipids, leads to cell death and multi-organ damage. While neurodegeneration is a major cause of morbidity and mortality in NPC disease, there are cases where death occurs within about the first six months of life from either liver failure or severe pulmonary dysfunction [1].

Although there is currently no effective treatment for NPC disease, a variety of agents has been evaluated primarily in animal or in vitro models, with 2-hydroxypropyl- β -cyclodextrin (2HP β CD) showing the best efficacy thus far [19–24]. In a particularly definitive study it was demonstrated that continuous infusion of 2HP β CD into the ventricular system of *Npc1*^{-/-} animals between 3 and 7 weeks of age normalized the biochemical abnormalities and completely prevented the expected neurodegeneration [25]. Earlier studies established that weekly subcutaneous administration of 2HP β CD to *Npc1*^{-/-} mice, starting at 7 days of age, nearly normalized hepatic and whole animal cholesterol pools and prevented development of liver disease [26]. While there was also a slowing of cerebellar neurodegeneration and an increase in lifespan, systemic 2HP β CD treatment had little to no effect on the development of progressive pulmonary disease [26]. Subsequent studies further demonstrated lack of impact of systemically administered 2HP β CD on a number of parameters of lung function and pathology in the *Npc1*^{-/-} mouse [27]. There is nothing intrinsically different about UC sequestration in the lungs in NPC1 or NPC2 deficiency because NPC2 replacement therapy in a mouse model of NPC2 disease resulted in a striking reduction in the cholesterol content of several organs including the lungs [28]. Rather, the inaction of 2HP β CD in the NPC lung may simply reflect lack of penetrance at a cellular level. Irrespective of whether this is the case, those studies revealed the paucity of published information about how NPC1 or NPC2 deficiency impacts lung cholesterol metabolism, particularly in early stage disease. In one of our initial studies in the *Npc^{nih}* mouse model we found that even in 1-day old *Npc1*^{-/-} pups the cholesterol concentration in most organs, including the lungs, was elevated [29]. More recently, a murine model of infantile NPC1 deficiency has been described but lungs were not included in the tissues examined [30].

The present studies represent the first systematic evaluation, in quantitative terms, of the ontogenic changes in lung mass, lipid composition, rates of cholesterol and fatty acid synthesis, and also histology in mice with NPC1 and NPC2 deficiency.

2. Materials and methods

2.1. Animals and diets

Control (*Npc1*^{+/+} and *Npc2*^{+/+}) and mutant (*Npc1*^{-/-} and *Npc2*^{-/-}) mice were generated from respective heterozygous breeding stock on a pure BALB/c background. The NPC1 mice (*Npc1^{nih}*) originated from a colony at the National Institutes of Health (Dr. Peter Pentchev),

while heterozygous *Npc2* breeding stock was kindly provided by Dr. Peter Lobel. Depending on the age at which they were to be studied, the mice were genotyped anywhere from 6 to 19 days of age. Unless studied beforehand, all mice were weaned at 19 to 21 days, and thereafter were fed ad libitum a cereal-based, low-cholesterol (0.02% cholesterol, 4% total fat, w/w) diet (No. 7001; Harland Teklad, Madison, WI). They were group-housed in plastic colony cages in rooms with alternating 12-h periods of dark and light and were studied in the fed state at the end of the dark phase. Unless otherwise stated there were comparable numbers of males and females in each group. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Southwestern Medical Center.

2.2. Changes in lung weight as a function of age and *Npc1* genotype

In the course of multiple projects with the *Npc1* mouse model we recorded lung weights in a total of 188 untreated animals (94 *Npc1*^{+/+} and 94 *Npc1*^{-/-}) over the age range of 8 to 80 days. The lungs were carefully excised, rinsed in physiological saline, blotted and weighed. In some of the mice studied in the age range of 57–60 days, the weight of each lobe (one on the left, and the diaphragmatic, apical, azygous, and cardiac on the right) was recorded.

2.3. Measurement of lung total cholesterol content and proportion of cholesterol present in esterified and unesterified form

Lung total cholesterol content was measured in *Npc1*^{-/-} mice and their matching *Npc1*^{+/+} controls at 8, 19, 29, 35, 49, 57, 63, and 70 days of age. For the *Npc2* mice such measurements were made at 70 days only. The total cholesterol concentration in the lungs (mg/g wet tissue weight) was measured by gas chromatography using stigmasterol as an internal standard [31]. The cholesterol concentration was multiplied by the respective lung weight, to give whole lung cholesterol content (mg/organ). In one study lungs were harvested specifically for measuring the proportion of the total cholesterol present in esterified and unesterified form. Lungs from *Npc1* mice (57–63 days old) and *Npc2* mice (70–80 days old) were weighed and extracted in chloroform:methanol (2:1 v/v) containing radiolabeled esterified and unesterified cholesterol as internal standards. The esterified and unesterified cholesterol fractions in these extracts were separated by column chromatography and quantitated as described [32].

2.4. Measurement of rates of cholesterol and fatty acid synthesis lungs in vivo

The details of the methods for these measurements are given elsewhere [31]. The ages of the *Npc1* mice used in this study were 8, 23, 52, and 70 days. The mice were given an i.p. injection of [³H]water (~2 mCi /g body wt), and after 1 h, were anesthetized and exsanguinated. The lungs were removed, rinsed and weighed. They were then saponified and their content of radiolabeled digitonin-precipitable sterols (DPS) was measured [31]. The rate of cholesterol synthesis was expressed as the nmol of [³H]water incorporated into DPS per h per whole organ. After the labeled sterols were extracted, the saponified samples were acidified and extracted with hexane. Aliquots of the organic phase were taken for measurement of [³H]-fatty acid content. Rates of fatty acid synthesis were expressed in the same units as for sterol synthesis. For the *Npc2* mice, the rates of lung sterol and fatty acid synthesis were measured at 70 days only.

2.5. Measurement of lung phospholipid and triacylglycerol content

These measurements were made in *Npc1*^{-/-} and matching *Npc1*^{+/+} mice at 8, 25, 50, and 70 days of age. They were not the same animals as those used for the determination of either the lung cholesterol content, or the lung cholesterol and fatty acid synthesis rates. After excision, the lungs were rinsed, weighed and added to chloroform:methanol (2:1 v/v).

Procedural losses were corrected for by internal standards. These were either [^{14}C]triolein (American Radiolabeled Chemicals, Inc., St. Louis, MO), or L- α -dipalmitoyl,[choline-methyl- ^3H]-phosphatidylcholine (Perkin Elmer NEN Radiochemicals, Waltham, MA). An aliquot of this extract was then dried, dissolved in hexane:tert-butyl methyl ether (100:1.5 v/v) and run over a Sep-Pak RC cartridge (500mg)(Waters Corp., Milford, MA) as described elsewhere [33]. After elution of the cholesteryl esters, the solvent was changed to hexane:tert-butyl methyl ether (96:4 v/v) for elution of triacylglycerols. Finally, the eluting solvent was changed to tert-butyl methyl ether:methanol:ammonium acetate (pH 8.6) (5:8:2 v/v/v) to obtain the phospholipid fraction. This fraction, as well as the one containing the triacylglycerols, was dried and redissolved in 10 ml of chloroform:methanol (2:1 v/v). Aliquots of these solutions were used for quantitation of total triacylglycerol content using Infinity Triglycerides Liquid Stable Reagent (Thermo Fisher Scientific, Inc., Middletown, VA) [34], or of total phospholipid content by a colorimetric assay using malachite green as described elsewhere [35]. In this method all the phospholipids are digested using perchloric acid at high temperature to liberate phosphorous on a mole per mole basis. It provides a measure of total phospholipid content and not of the proportion of phospholipid species that are present. This requires the use of other techniques including LC/MS/MS as described below. The lung triacylglycerol data were expressed as mg/organ while those for phospholipid were calculated as $\mu\text{mol/organ}$.

2.6. Determination of lung and liver phospholipid composition

The whole lungs and aliquots of livers from three *Npc1*^{-/-} mice and three matching *Npc1*^{+/+} controls at 49 to 53 days of age were weighed and extracted in chloroform:methanol (2:1 v/v). These pooled extracts were dried under nitrogen and shipped on dry ice to Avanti Polar Lipids, Inc. (Alabaster, AL) where the phospholipid composition was determined by LC/MS/MS. The proportion of each phospholipid detected was expressed as a fraction of the total phospholipid content. For all the phospholipids except for sphingomyelin, trace quantities of their lyso form were detected. Given the uncertain origin of these lyso phospholipids they were simply added to the mass of the parent phospholipid that was measured.

2.7. Lung histology

Lungs were perfused with 10% buffered formalin and excised. The tissue was fixed in 10% buffered formalin and embedded in paraffin. These blocks were then sectioned and stained with hematoxylin and eosin (H&E). For the *Npc1* mice, lung histology was obtained at 28, 49, and 70 days of age with lungs being taken from three *Npc1*^{-/-} and three *Npc1*^{+/+} mice at each of these ages. For the NPC2 mice, lung histology was carried out at only 70 days in two *Npc2*^{-/-} and two *Npc2*^{+/+} mice.

2.8. Analysis of data

All data are reported as the mean \pm 1 SEM in the specified number of individual animals. Differences between mean values were tested for statistical significance ($p < 0.05$) by the two-tailed Student's *t*-test assuming equal variance.

3. Results

Although several mouse models of *Npc1* deficiency have been described [2, 11, 14, 30] all of our studies to date have used *Npc1*^{mihi} mice maintained on a low-cholesterol basal rodent chow diet. These mice exhibit a marked increase in whole body cholesterol synthesis and content [29, 36]. While all organs show elevated concentrations and rates of synthesis of cholesterol, these changes are particularly pronounced in the liver [36]. Consequently the *Npc1*^{-/-} mice manifest an age-related increase in plasma ALT and AST activities [34].

These changes are accompanied by progressive neurodegeneration which leads to a shortened life span of around 80 to 90 days [13, 26]. The features of *Npc2*^{-/-} mice parallel those lacking *Npc1* but their lifespan is generally longer, depending partly on the strain of mouse [12, 28, 37].

3.1. *Npc1*^{-/-} mice show a significant increase in lung mass starting before weaning

At 8 days after birth the weight of the lungs in the *Npc1*^{-/-} mice (0.080 ± 0.003 g, n=10) was about the same as that in matching *Npc1*^{+/+} controls (0.079 ± 0.003 g, n=12) (Fig. 1A). However, at all ages after 8 days the lung mass in the *Npc1*^{-/-} mice was consistently greater than in their *Npc1*^{+/+} controls. From the data in Fig. 1A it can be calculated that across the age span from 19–22 to 77–80 days, the average weight of the lungs in the *Npc1*^{-/-} mice (0.165 ± 0.010 g) was significantly higher than in the matching *Npc1*^{+/+} controls (0.134 ± 0.006 g, $p < 0.05$). At least in mice in the age range of 57–60 days, the greater lung mass in the mutants was not confined to any one lobe although the bulk of the additional tissue was in the left and diaphragmatic lobes (Fig. 1B).

3.2. Marked increase in lung total cholesterol concentration in *Npc1*^{-/-} and *Npc2*^{-/-} mice is due only to the unesterified component

Before carrying out detailed measurements of genotypic and ontogenic changes in lung cholesterol content and synthesis, and other parameters of lipid metabolism, we first quantitated the magnitude of the increase in the total cholesterol concentration (mg/g) in the lungs of both *Npc1*^{-/-} and *Npc2*^{-/-} mice exhibiting early clinical symptoms of disease. Furthermore, we also wanted to confirm that it was expansion of the unesterified cholesterol component that was driving the elevation in cholesterol concentration in the lungs of both models. The data show unequivocally that the 2.7-fold and 2.4-fold increase in total cholesterol concentrations in the *Npc1*^{-/-} and *Npc2*^{-/-} mice respectively, was entirely accounted for by unesterified sterol (Fig. 2). These data also make the point that while changes in whole lung cholesterol content (mg/organ) described below reflect both greater tissue mass and cholesterol concentrations, it is the latter that accounts for much of the expansion of the lung cholesterol pool in NPC deficiency. One earlier study with *Npc1*^{-/-} mice showed their hepatic esterified cholesterol concentrations were not elevated unless they were fed a high cholesterol diet [36]. However, another study using *Npc1*^{-/-} primary hepatocytes did find some accumulation of cholesteryl ester in these cells [38].

3.3. Ontogenic changes in lipid composition and synthesis rates in the lungs of *Npc1*^{-/-} mice are not uniform for all the major classes of lipids

The data in Fig. 3A show lung weights relative to body weights in *Npc1*^{-/-} and *Npc1*^{+/+} from 8 to 70 days which was generally the age range over which various parameters of lung cholesterol and lipid metabolism were measured. It should be noted that after about 49 days of age the genotypic difference in relative lung weight becomes exaggerated because of the steady decrease in the body weight of the *Npc1*^{-/-} mice in late stage disease.

In the 8-day old mutant mice a higher lung cholesterol content was already apparent (Fig. 3B). At 23 days lung cholesterol content in the mutants was 3.3-fold more than in the matching *Npc1*^{+/+} mice (Fig 3B). This difference remained at about 3-fold until 70 days of age when it increased to 3.7-fold. While the data are not shown, in 80-day old *Npc1*^{-/-} mice whole lung cholesterol was 4.3-fold higher than in their *Npc1*^{+/+} littermates (3.2 ± 0.2 vs 0.75 ± 0.05 mg/organ). Although measured at fewer time points, the genotypic differences in lung phospholipid content nevertheless generally followed the same pattern as those found for cholesterol. At both 49 and 70 days lung phospholipid content in the *Npc1*^{-/-} mice was about 2.7- to 2.8-fold higher than in matching *Npc1*^{+/+} controls (Fig. 3C). In sharp contrast, in the case of lung triacylglycerol content, the values for the *Npc1*^{-/-} mice were consistently

less than those for the *Npc1*^{+/+} animals from about the time of weaning onwards (Fig. 3D). In addition to the lipid composition measurements, separate groups of mice at 8, 23, 52, and 70 days of age were used for the determination in vivo of the rates of cholesterol and fatty acid synthesis in lung, expressed on a whole organ basis. At just 8 days of age lung cholesterol synthesis in the mutants exceeded that in the *Npc1*^{+/+} controls by 4.5-fold, and remained more than 2-fold higher at all the other age points (Fig. 3E). In marked contrast there was no discernible genotypic difference in lung fatty acid synthesis at any age (Fig. 3F).

3.4. Lung phospholipid composition did not show a genotypic difference but in the livers of the *Npc1*^{-/-} mice the proportion of sphingomyelin was markedly elevated

Given that phospholipids are the most abundant lipid class in the lung, and also that other investigators had earlier reported an elevation in hepatic sphingomyelin levels in the livers of *Npc1*^{-/-} mice [39], we carried out a phospholipid composition analysis by LC/MS/MS in pooled lipid extracts from the lungs and livers of *Npc1*^{-/-} and *Npc1*^{+/+} at 49–53 days of age. There were no discernible genotypic differences in lung phospholipids, but in the liver the relative proportion of sphingomyelin (SM) in the *Npc1*^{-/-} mice was 7.3-fold higher than in the *Npc1*^{+/+} controls (data not shown).

3.5. Histology of the lungs revealed an increasing presence of alveolar lipid-laden macrophages in the *Npc1*^{-/-} mice as they age

The finding of marked changes in the lipid composition of the lungs in the *Npc1*^{-/-} mice starting well before adulthood prompted an examination of lung histology in *Npc1* mice of both genotypes at 28, 49, and 70 days by H&E staining. In the *Npc1*^{+/+} mice over this age range no macrophage infiltration was observed (Fig. 4A–C). However, in marked contrast, lungs from the 28-day old *Npc1*^{-/-} mice revealed the presence of a few scattered, yet abnormal appearing lipid-laden macrophages within the alveoli (Fig. 4D). This feature was much more evident at 49 days (Fig. 4E), and at 70 days (Fig. 4F). In both cases an increasing presence of alveolar lipid-laden macrophages is readily discernible.

3.6. Lung mass, cholesterol metabolism and histology in *Npc2*^{-/-} mice show changes comparable to those manifest in *Npc1*^{-/-} mice

The striking age-related changes in lipid metabolism and histology in the lungs of the *Npc1*^{-/-} mice prompted an abbreviated investigation of these same parameters in the lungs of *Npc2*^{-/-} mice at the single age point of 70 days. In our earlier studies comparing *Npc1* and *Npc2* mice, both on the BALB/c background, the median lifespan of the *Npc2*^{-/-} mice (146 days) was notably longer than that of the *Npc1*^{-/-} mice (89 days) [12]. Thus, at 70 days, *Npc2*^{-/-} mice have less advanced disease than those with NPC1 deficiency at that age. Nevertheless the relative lung weight in the *Npc2*^{-/-} mice at 70 days ($0.86 \pm 0.04\%$) was almost 50% more than that in the *Npc2*^{+/+} ($0.58 \pm 0.02\%$) controls (Fig. 5A), but below the relative lung weight seen in 70-day-old *Npc1*^{-/-} mice ($1.1 \pm 0.1\%$) (Fig. 3A). In the *Npc2*^{-/-} mice lung cholesterol content was elevated 2.7-fold (Fig. 5B) and the rate of cholesterol synthesis was 61% greater than in their *Npc2*^{+/+} controls (Fig. 5C). There was no corresponding genotypic difference in the rate of fatty acid synthesis in the lung (Fig. 5D). H&E staining showed a clear difference histologically in the lungs of the *Npc2*^{+/+} (Fig. 5E) and *Npc2*^{-/-} (Fig. 5F) mice. In the latter case lipid-laden macrophages were evident as seen in the lungs of the *Npc1*^{-/-} mice.

4. Discussion

Four main conclusions can be drawn from the current work. The first of these relates to changes in the mass of the lungs from early to late stage disease. An early publication

describing lipid metabolism in the *Npc1*^{-/-} mouse (then known as the NCTR-BALB/c mouse) noted a 2.3-fold higher relative lung weight in the mutants at about 70 days of age [3]. A comparable genotypic difference was seen in the relative lung weights in the 70 day old *Npc1*^{-/-} mice in the present studies (Fig. 3A). It should be emphasized that such relative organ weight data in late stage NPC disease can be difficult to interpret because, after about 7 weeks of age, the *Npc1*^{-/-} mice show an accelerated loss of body weight. Hence, relative organ weights, whether they be for lung, liver or spleen rise, but mostly this is not due to a continuing expansion of organ mass. The more important point to be made here, however, is that, although hepatosplenomegaly is a hallmark of NPC disease, there is also a decisive increase in lung mass. In the present studies this was clearly the case for the *Npc1*^{-/-} mice (Fig. 1), and for the *Npc2*^{-/-} mice as well (Fig. 5A). What the present studies also reveal is that the expansion in lung mass in the *Npc1*^{-/-} mice is clearly evident from about the time of weaning. It is noteworthy that in a young NPC2-deficient human subject a marked increase in lung weight was reported [40].

The second conclusion concerns the striking ontogenic differences in lung cholesterol content and synthesis which were evident starting in early neonatal life. As documented by many laboratories, and as again confirmed here (Fig. 2), the increase in tissue cholesterol content in all organs, including the lungs, in both NPC1 and NPC2 disease reflects an expansion of the unesterified cholesterol fraction [3, 13, 14, 26]. By 50 days of age the lung cholesterol content in the *Npc1*^{-/-} mice was 2.8-fold more than in matching *Npc1*^{+/+} controls. This cholesterol sequestration in the lungs is detectable as early as one day after birth in the *Npc1*^{-/-} mice [29]. It was therefore not surprising to find that, at just 8 days of age, cholesterol synthesis in the lungs of the mutant mice was already four-fold above normal. The rapidly developing lung in the neonate has a critical need for cholesterol both for tissue growth and surfactant production. Ordinarily, most of this need would be met through the uptake of lipoproteins by receptor-mediated and bulk-phase endocytosis. When cholesterol entering tissues through these pathways becomes sequestered in the late E/L compartment because of either NPC1 or NPC2 deficiency this is compensated for in all tissues, including the lungs, by an increase in de novo sterol synthesis [8, 36]. This effect was selective for cholesterol synthesis because the rate of fatty acid synthesis in the lungs of the *Npc1*^{-/-} mice was about the same as that in the *Npc1*^{+/+} controls at all ages (Fig. 3F).

The next conclusion relates to the divergence in the genotypic- and age-related changes in lung phospholipid (Fig. 3C) and triacylglycerol (Fig. 3D) content. For these data, as well as those for cholesterol (Fig. 3B), it should be emphasized that since the lungs were not lavaged before harvesting, the tissue lipid composition partially reflects that of the residual surfactant. However, data for other types of murine models of pulmonary dysfunction have shown that the phospholipid composition of alveolar lavage fluid mirrors that of the lung tissue [41–43]. Moreover, the total amount of phospholipid found in alveolar lavage fluid was reported to be about 10% of that contained in the lung tissue and fluid combined [41]. Similarly, the cholesterol content of alveolar lavage is very small compared to that of lung tissue [44]. It remains to be determined why the total phospholipid content of the lungs in the mutants rises by about the same magnitude, and follows a similar pattern of age-related changes as seen for cholesterol. Based on studies in primary hepatocytes from *Npc1*^{-/-} mice a probable explanation is that there is an increase in phospholipid synthesis [38]. If so, this would potentially act as a compensatory mechanism to balance the increase in membrane cholesterol content thereby helping to preserve membrane structure and function. Alternatively, there may be a reduction in surfactant phospholipid degradation [45].

The lung triacylglycerol (TAG) content data (Fig. 3D) are very different from those found for phospholipid (Fig. 3C). In the pioneering work of Pentchev et al. [2], a dramatic depletion of tissue TAG content in several organs, including lung, was found in 72-day old

Npc1^{-/-} mice. In our earlier studies with 56-day old *Npc1*^{-/-} mice we described a marked reduction in hepatic TAG content [34]. Several subsequent studies with hepatocytes have confirmed this finding [38, 46, 47]. One of these concluded that the diminished TAG levels resulted from the accelerated rate of utilization of acetate for cholesterol synthesis [47]. However, the measurement of TAG synthesis in primary hepatocytes from *Npc1*^{-/-} mice showed that it is not significantly altered [38]. In the case of the lung we show here that lung TAG content in the *Npc1*^{-/-} mice is normal at birth but declines markedly thereafter. Clearly this is not due to a reduction in fatty acid synthesis (Fig. 3F).

The fourth conclusion relates to the type of cells in the lungs that are affected in NPC disease. Using H&E stain, abnormal alveolar macrophages are seen as early as 28 days and accumulate over time. Surprisingly, infiltration with intra-alveolar lipidladen macrophages seen in 70 day old *Npc1*^{-/-} mice is equally as severe as in *Npc2*^{-/-} mice of the same age. This poses a dilemma because case reports of NPC2 patients are dominated by lung involvement characterized by alveolar foamy macrophage accumulation [40, 48, 49]. On the other hand, our observations are similar to those reported in a newly published study in both mouse and feline models of NPC1 and NPC2 disease [45]. Further evaluation with electron microscopy in our laboratory has identified macrophages with lipid accumulation and vacuolar inclusions infiltrating the lung parenchyma and enlarged, pleomorphic lamellar bodies within the Type II pneumocytes (unpublished data). These findings are consistent with those of the newly reported work of Roszell et al [45], as well as of an earlier study [50].

The final point to be made concerns the directions that future research on lung disease in NPC1 and NPC2 deficiency might reasonably follow. One of these is the delineation of changes in gene expression levels for a constellation of parameters including surfactant production, cholesterol metabolism, phospholipid synthesis and transport, apoptosis, macrophage infiltration, and inflammation. Ideally, such measurements should be carried out in early and late stage disease in one of the murine models for NPC deficiency as defined by the ontogenic data presented here. Another area of investigation clearly needed has to do with the cause(s) of the failure of systematically administered 2HPβCD to mobilize unesterified cholesterol sequestered in the E/L compartment in the various types of alveolar and epithelial cells in the lung parenchyma. If limited penetrance is the underlying cause then new technical approaches for delivering finely aerosolized 2HPβCD through inhalation will need to be designed and tested. The existing literature on pulmonary dysfunction in humans with NPC disease [40, 48, 49, 51–53] clearly underscores the need for more research at both a basic and clinical level.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
bw	body weight
E/L	endosomal/lysosomal
2HPβCD	2-hydroxypropyl-β-cyclodextrin

Npc1	Niemann-Pick C1
Npc2	Niemann-Pick C2
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SM	sphingomyelin
TAG	triacylglycerol
UC	unesterified cholesterol

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Highlights

- NPC1-deficient mice have increased lung mass starting during neonatal development
- Lung content of cholesterol and phospholipid in *npc1*^{-/-} mice increase with age
- Lungs of *npc1*^{-/-} mice show increase in synthesis of cholesterol but not fatty acids
- Lungs of *npc1*^{-/-} mice show an increase in lipid-laden macrophages in the alveoli
- Effects of NPC2 deficiency on lung lipid metabolism resemble those in *npc1*^{-/-} mice

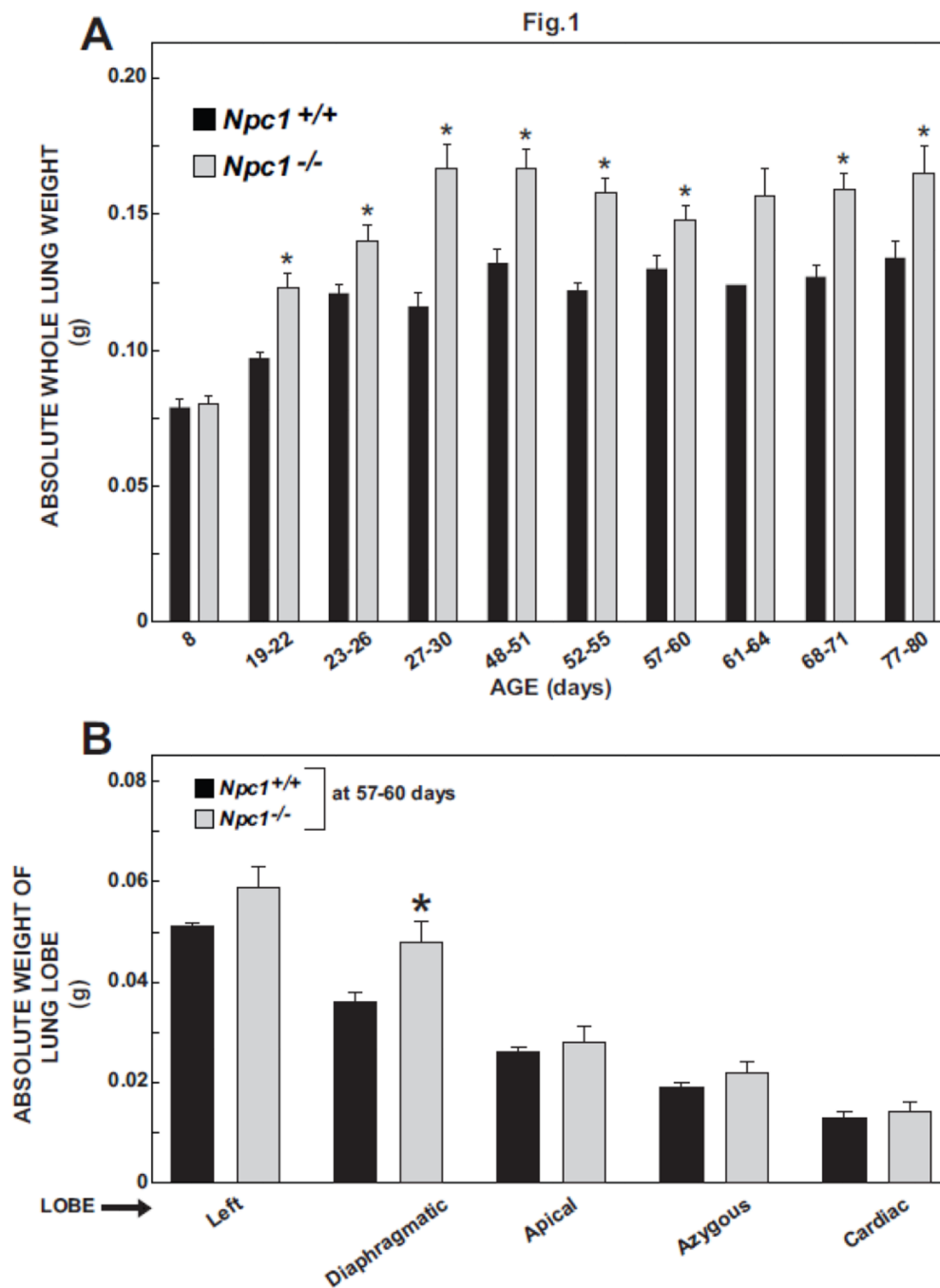


Fig. 1. Absolute whole lung weights in *Npc1*^{+/+} and *Npc1*^{-/-} mice over the age range of 8 to 80 days, and weights of individual lung lobes in *Npc1*^{+/+} and *Npc1*^{-/-} mice at 57–60 days. The data for lung weights from all the present experiments were pooled with those from previous projects in this laboratory using *Npc1* mice. Except for the 8-day time point, the data are grouped for animals over successive spans of 3 days each (A). Lung weights were obtained from a total of 188 mice (94 for each genotype). The numbers of mice at each age varied considerably. At the 8, 19–22, 27–30, 48–51, 52–55, and 68–71 day time points there was a minimum of 9 mice of each genotype. For all other points there was a minimum of 4 mice of

each genotype except in the group at 61–64 days where there were just two *Npc1*^{+/+} mice. Separate groups of mice in the age range of 57–60 days were used for the measurement of the weights of individual lobes (B). These data are from 4 mice of each genotype. All values represent the mean \pm 1 SEM. The asterisk denotes that the value for the *Npc1*^{-/-} mice is significantly different from that for the *Npc1*^{+/+} controls at that particular age ($p < 0.05$).

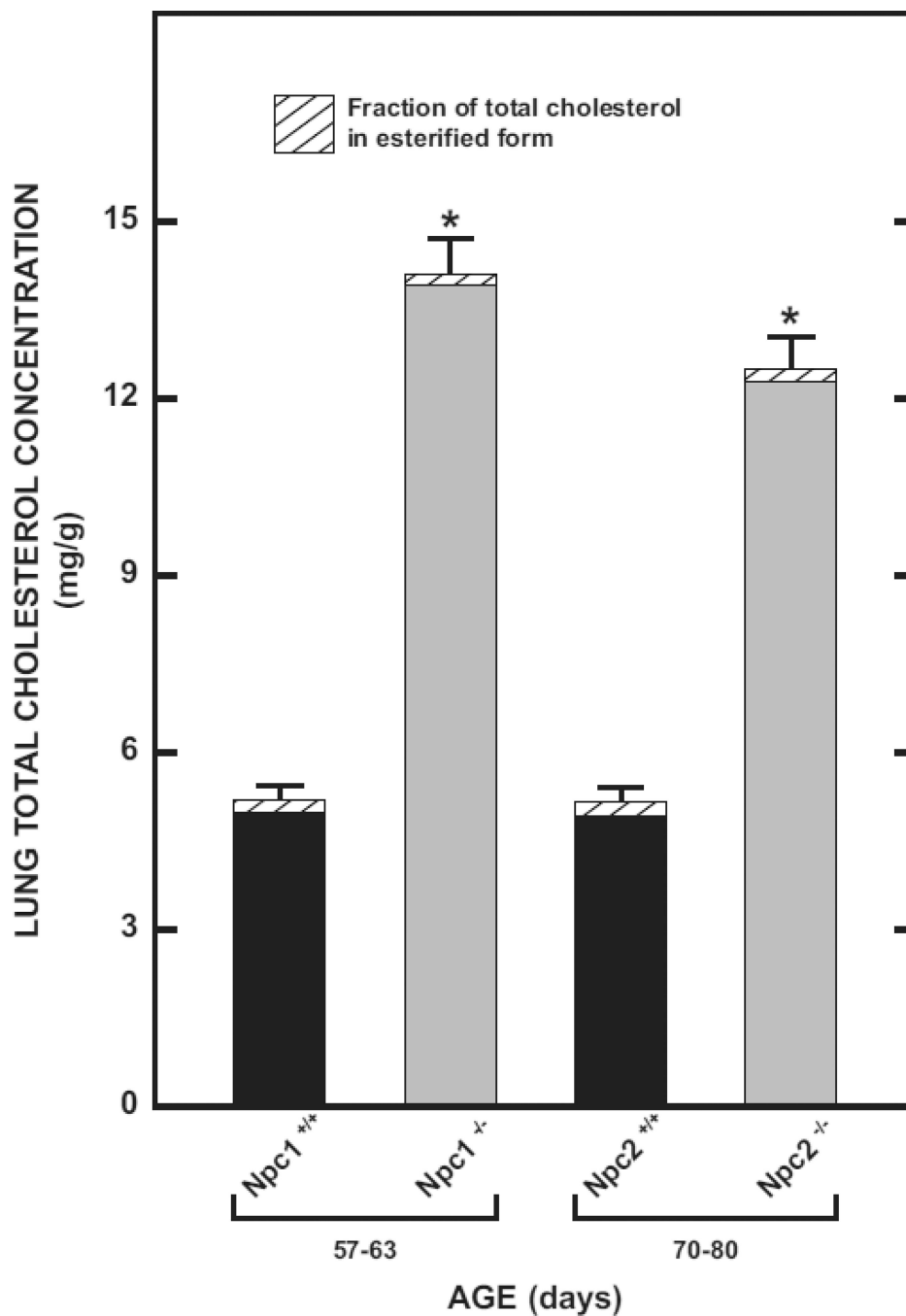


Fig. 2. Total, unesterified, and esterified cholesterol concentrations in the lungs of *Npc1*^{+/+}, *Npc1*^{-/-}, *Npc2*^{+/+}, and *Npc2*^{-/-} mice. The unesterified and esterified cholesterol concentrations were measured as described in Materials and Methods and summed to give the total cholesterol concentration. In each histogram the solid shading represents the level of unesterified cholesterol. Values represent the mean \pm 1 SEM of measurements in four mice in each group. The asterisk denotes that the values for the *Npc1*^{-/-} and *Npc2*^{-/-} mice are significantly different from the value for the *Npc1*^{+/+} and *Npc2*^{+/+} mice, respectively ($p < 0.05$).

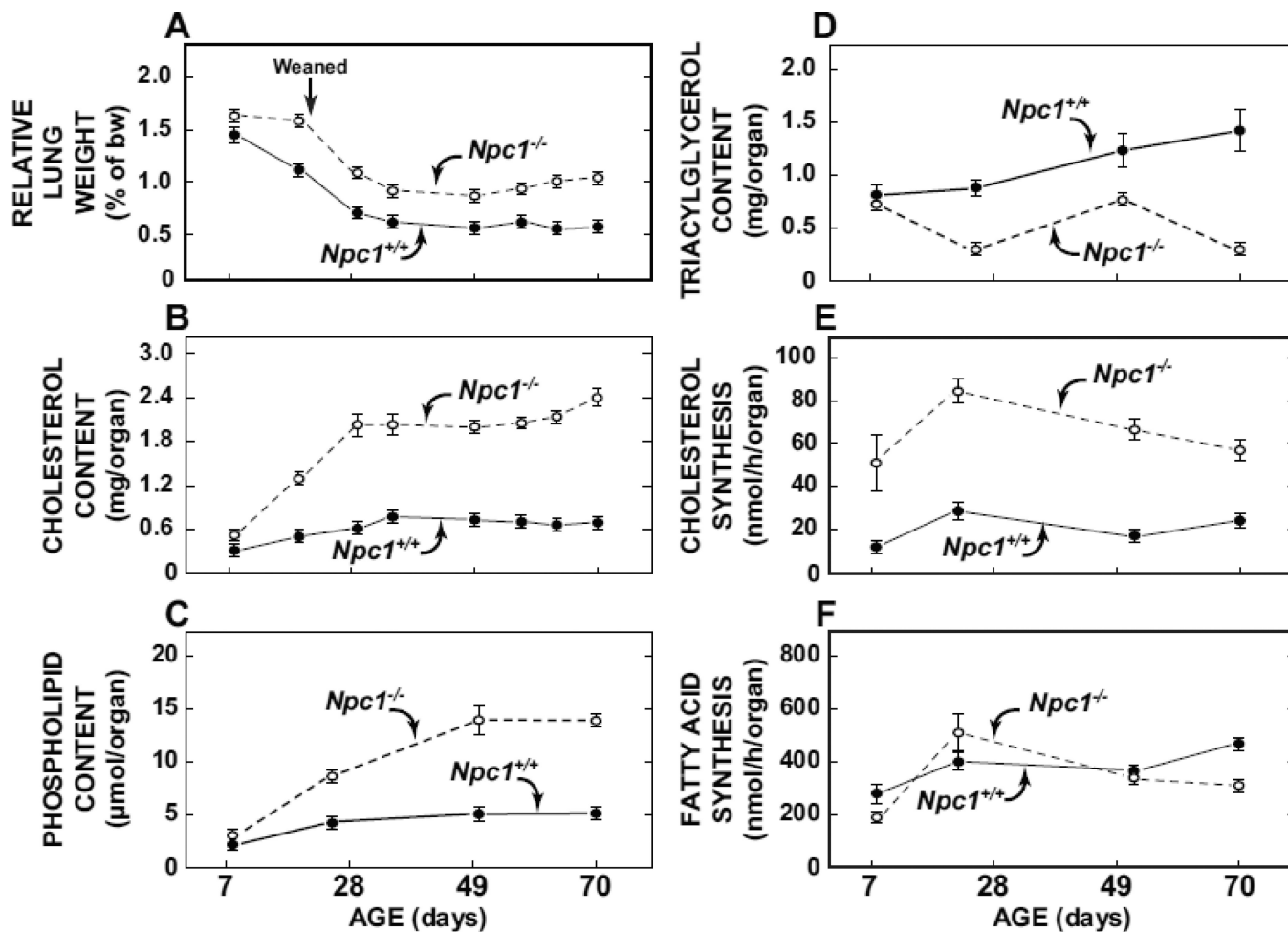


Fig. 3. Age-related changes in the relative weight, lipid composition, and synthesis rates of cholesterol and fatty acid in the lungs of *Npc1*^{+/+} and *Npc1*^{-/-} mice. The data for relative lung weight (A) and whole lung cholesterol content (B) were obtained from mice at 8, 19, 29, 35, 49, 57, 63 and 70 days of age. For the measurement of lung phospholipid (C) and triacylglycerol (D) content tissue was taken at only four ages (8, 25, 50, and 70 days). This was also the case for the measurement of the rates of lung cholesterol synthesis (E) and fatty acid synthesis (F) (8, 23, 52, 70 days). These rates were measured in vivo using [³H]water as described in Materials and Methods. The groups of mice used for this procedure were separate from those used for the chemical composition assays. For all parameters the values at each time point represent the mean ± 1 SEM of measurements in a minimum of four mice.

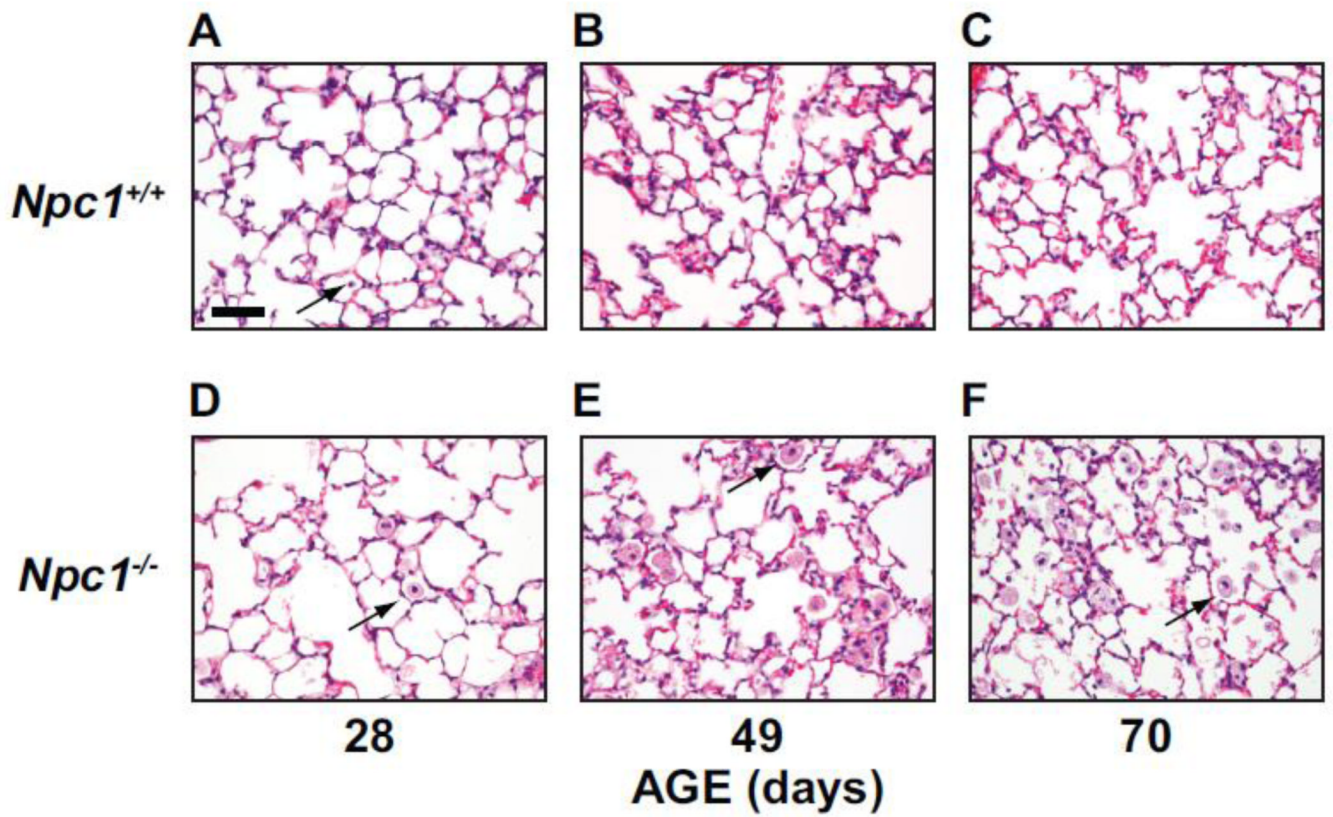


Fig. 4. Representative histological sections of lung tissue from *Npc1*^{+/+} and *Npc1*^{-/-} mice at three different ages. (A) The arrow points to a normal alveolar macrophage in the *Npc1*^{+/+} mouse. (D-F) the arrows point to abnormal lipid-laden macrophages within the alveoli of *Npc1*^{-/-} mice. The scale bar equals 100 μ m (A), and all six images are at the same magnification ($\times 400$). For each age, lung histology was done on three mice of each genotype.

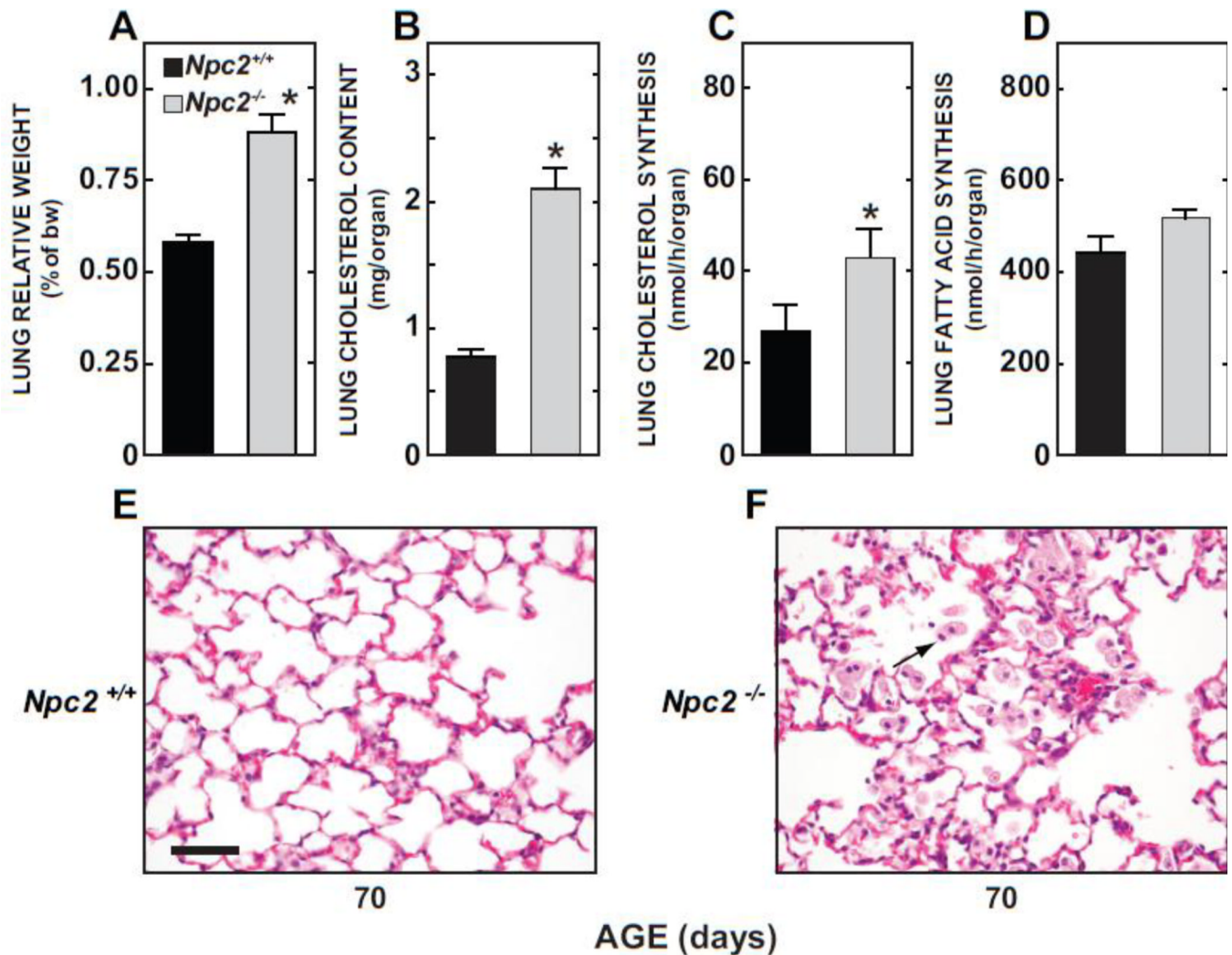


Fig. 5. Lung relative weight, cholesterol content, rates of cholesterol and fatty acid synthesis and histology in *Npc2*^{+/+} and *Npc2*^{-/-} mice at 70 days of age. (A–D) the organ weight and metabolic data were obtained from a single study using six *Npc2*^{+/+} and six *Npc2*^{-/-}, all at 70 days of age. For all of these parameters the values are mean ± 1 SEM. The asterisk denotes a statistically significant difference ($p < 0.05$). Separate mice were used to obtain lungs for histology. The two lower images show representative histological sections of lung tissue in *Npc2*^{+/+} (E) and *Npc2*^{-/-} (F) mice. The arrow points to a lipid-laden macrophage within the alveoli of the *Npc2*^{-/-} mouse. The scale bar equals 100 μm (E) and both images are at the same magnification (×400).