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# Cell and Tissue Heterogeneity in Peroxisomal Patients

## Introduction

Because in congenital peroxisomal disorders the defect is genetic, it is generally assumed to be expressed in all tissues, for ex. in liver parenchyma and cultured skin fibroblasts, and in all liver parenchymal cells alike. In this report we show that there are many examples of differences between fibroblasts and liver, but more remarkably between individual cells in the same liver. The latter condition is more appropriately named mosaicism, of which we describe five cases, in addition to a sixth one reported elsewhere [27].

In recent publications the microscopic, cytochemical and morphometric alterations of peroxisomes in congenital disorders, including the visualization of ghosts in human liver, as well as in several acquired human diseases, were reviewed [20, 57, 61, 62]. For the purpose of this report, the main data are summarized.

## Overview of previous cytochemical data

In Zellweger cerebro-hepato-renal syndrome, and in most patients with infantile Refsum disease, hepatic peroxisomes are absent, but catalase is visibly localized in the cytoplasm. Cytoplasmic localization of other peroxisomal enzymes (D-amino acid oxidase, glycolate oxidase) in Zellweger livers could not be demonstrated by cytochemistry [30], probably because the proteins were not immobilized by proper fixation.

Immunolocalization of the 70 kDa peroxisomal membrane protein in these livers has not revealed the existence of "ghosts" or other structures [27, 62], in contrast to several reports about such ghosts in cultured fibroblasts. Recently in this laboratory a 43 kDa human peroxisomal membrane protein was localized in the liver of eight patients with multiple peroxisomal defects (as diagnosed biochemically), by use of antibodies prepared by Dr Manuel Santos; the liver parenchyma was devoid of any structure containing peroxisomal enzymes [27]. Two types of labelled organelles were observed: dense-cored, and 'empty' vesicles; they were very rare in comparison to the frequency of normal

peroxisomes. Both types co-occured in four patients. In a classical Zellweger patient only the 'empty' type was found, measuring 0.2-0.3 $\mu$m. One patient diagnosed as a mild Zellweger syndrome had 'empty' vesicles in the majority of the parenchymal cells; but in addition other, rare cells were encountered showing numerous, small (0.2-0.3 $\mu$m) peroxisomes with catalase, thiolase and AGT. This mosaic is described by Espeel et al. [27]. In normal livers, the anti-43 kDa antibody exclusively labelled the peroxisomal membrane [27].

In most other $\beta$-oxidation defects, the liver contained enlarged peroxisomes (in oxidase- and thiolase deficiency, Zellweger-like and several variants presenting as NALD-like), with the exception of X-linked ALD (normal looking peroxisomes) and true NALD (rare and very small, dense-cored organelles) - [36, 38, 74]. Hughes et al. [38] mentioned that the dense- cored organelles appeared not to react for catalase activity. Enlargement of peroxisomes is also striking in RCDP [19, 39; also this report], in contrast to a recent claim [21].

In some patients enlarged hepatic peroxisomes contain alanine-glyoxylate aminotransferase and three $\beta$-oxidation enzyme proteins, but catalase is free in the cytoplasm [24, 62]. The peroxisomal localization of catalase activity and the three $\beta$-oxidation enzyme proteins is compatible with a functional defect in vivo, i.e. accumulation of VLCFA and abnormal bile acids [26, 76].

Peroxisomes in hyperpipecolic aciduria are the subject of an apparent controversy : Challa et al. [12] mentioned their presence in the liver biopsies of two patients, without showing a photograph however; in cultured fibroblasts of an other HPA patient, Wanders et al. [78] found cytoplasmic catalase, while Wiemer et al. [79] showed punctate fluorescence in these cells. We have studied the liver of two patients with increased pipecolic acid without VLCFA accumulation : both had catalase containing organelles (Poll-The & Roels). Therefore classification of HPA together with Zellweger syndrome in group I peroxisomal disorders appears unjustified.

In human fetal liver, vesicles devoid of catalase activity were seen prior to the appearance of this enzyme, which again preceded in time the $\beta$-oxidation enzymes [23, 25], giving evidence for the import of matrix proteins into pre-existing structures. In all human livers peroxisomal catalase concentration (activity per unit volume) differs from one peroxisome to the other, within the same cell. Free exchange of matrix proteins is therefore unlikely [58]. Continuities between peroxisomes such as described in animals during proliferation (partial hepatectomy or peroxisome proliferators) were not seen in human liver.

In many acquired diseases (viral, alcoholic and drug induced hepatitis, cirrhosis, cancer with or without liver metatasis) hepatic peroxisomes displayed secondary alterations, such as increased numbers or reduced catalase staining; enlargement was never observed [14, 15, 16, 17]. Inhibition of mitochondrial $\beta$-oxidation by chlorpromazine elicited peroxisomal proliferation in man [13]. The decrease of catalase activity in the presence of malignant tumors has been known since 1910 ([9], see [64]). Tumor xenografts in mice reduced catalase activity in liver and duodenal epithelium; fast-growing tumors, but not slow-

growing ones lead to smaller liver peroxisomes [20]. Tumor necrosis factor $\alpha$ probably is a mediator in this process, it also depresses $\beta$-oxidation capacity.

Enlarged peroxisomes were experimentally induced in rats by hypothyroidism [55], the opposite, i.e. smaller organelles, was seen after administration of thyroid hormones [31, 43]. We have proposed that import of matrix components is proportional to the surface area of the peroxisomal membrane, and this must lead to an inverse relationship between size and the concentration of enzymes as well as of substrates, and thus, functional capacity. This might be one causative factor in inborn disorders with enlarged peroxisomes [57, 58, 62].

## Methods

After immobilization of cell components by adequate fixation in buffered formaldehyde-calcium [64], followed, for immunocytochemistry, by glutaraldehyde, catalase activity was localized by staining with diaminobenzidine at pH 10.5 [65]; antigens of catalase, alanine-glyoxylate aminotransferase, acylCoA oxidase, trifunctional enzyme, keto-thiolase, and peroxisomal membrane proteins, were localized with protein A-gold, intensified with silver for light microscopy [24]. Fat insoluble in acetone, n-hexane or xylene is stained with Oil Red O or Sudan Black B in prefixed sections. Transparent polarizing inclusions were detected by light microscopy predominantly in macrophage lysosomes, and their trilamellar, regularly spaced sheets, by conventional electron microscopy [44, 59, 62]. Lysosomes were identified by localization of acid phosphatase activity with several substrates and capturing agents [59, 62].

## Results

Five patients with hepatic mosaicism are described. The first patient P, reported in detail by Mandel et al. [48] is now 9 y old and the son of consanguinous parents; he has a younger brother with a very similar presentation. Their neurodegenerative disease is accompanied by the impairment of multiple peroxisomal functions as shown biochemically. At the age of 8 y, liver sections stained for catalase activity showed typical granules in small areas of the parenchyma, but in most hepatocytes there were none. Instead the cytoplasm was dark indicating cytoplasmic catalase. The boundary between both types of regions was sharply delineated; there was no chess-board pattern. That the granules are peroxisomes was confirmed by electron microscopy; they had normal shape and size, including varying amounts of catalase reaction product as seen in most livers [48]. By immunostaining, 4 matrix enzymes: alanine-glyoxy late aminotransferase, acylCoA oxidase, thiolase and catalase, were also localized in these peroxisomes; in other regions a cytoplasmic anti-catalase label was seen. On immunoblots of liver homogenate, only traces of peroxisomal

$\beta$-oxidation enzyme proteins were detected. This was understood when the volume occupied by the peroxisome containing cells was estimated: by microscopical examination of some 30 samples from the biopsy, many of which had no peroxisomes, a mean volume of around 8% was calculated. However, fibroblasts cultured from two consecutive skin biopsies of this patient had entirely normal peroxisomal functions (VLCFA, *de novo* plasmalogen synthesis, dihydroxyacetone phosphate acyltransferase activity, particle-bound catalase). Other features in liver were in agreement with the peroxisomal origin of the illness [62]: enlarged macrophages containing abundant birefringent material as well as fat droplets insoluble in acetone, and ultrastructurally, numerous trilamellar sheets embedded in acid phosphatase activity inside angulate lysosomes [48]. Ghosts bearing the 43 kDa membrane protein were not observed in the cells devoid of normal peroxisomes.

The second patient, MS, of Dr Poggi's, has been reported [27A]. He is the 2y old son of unrelated parents. He presented with digestive symptoms (milk intolerance, failure to thrive) and evident neurological signs: severe mental

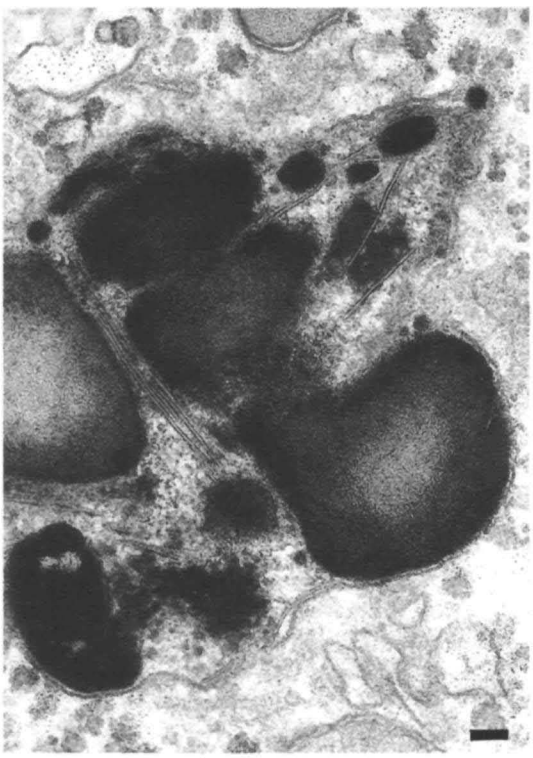

Fig. 1. Trilamellar inclusions (two electron dense equidistant lines bordering an electron lucent space) inside an angulate lysosome of a parenchymal cell of patient No. 2. They are evidence of a peroxisomal deficiency disorder. Lysosome also contains very dense lipid droplets of variable sizes which have resisted extraction by ethanol and embedding media. Scale bar = 0.1 micrometer.

retardation, major axial hypotonia but spastic hypertonia of the limbs, persisting archaic reflexes and myoclonic jerks indicating central motor lesions. Electromyography suggested an additional peripheral disturbance. Brain MRI showed a cortical and subcortical atrophy. There was no paresis however; visual evoked potentials had somewhat increased latency, but the child had eye contact and reacted by smiling. Biochemistry indicated multiple peroxisomal dysfunction: increased serum VLCFA and pipecolic acid, abnormal bile acids, but no phytanic acid. Remarkably, cultured fibroblasts had a normal oxidation of lignoceric acid. The liver biopsy gave convincing evidence of peroxisomal involvement by the presence of trilamellar structures in parenchymal lysosomes (Fig. 1) and fat insoluble in acetone-hexane in large PAS-positive macrophages; there were a few small polarizing inclusions. Moreover peroxisomes were absent in approx. 90% of the biopsy by visualization of catalase activity (Fig. 2, 4), and by immunocytochemistry of thiolase, AGT (Fig. 3) and catalase. The last enzyme was localized in the cytoplasm of these parenchymal cells without peroxisomes (Fig. 2). Membrane vesicles (ghosts) labelling for the 43 kDa

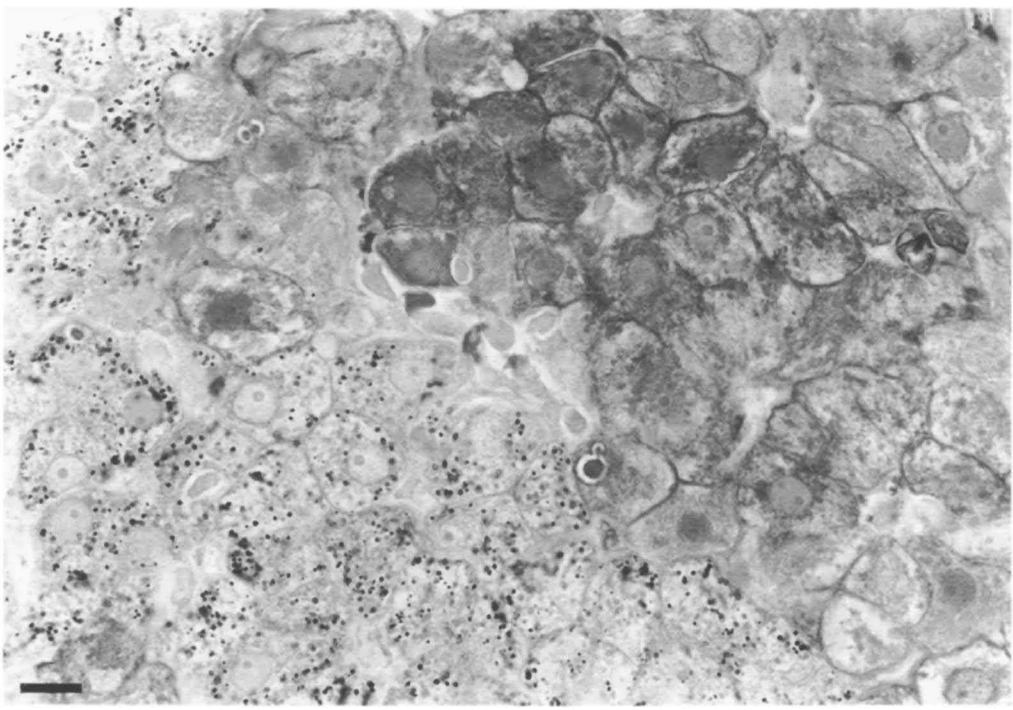

Fig. 2. Visualization of catalase activity in liver of patient 2. Photograph shows boundary between region of the parenchyma without peroxisomes but displaying dark cytoplasm due to catalase reaction product, and a group of cells with peroxisomes (which are seen as numerous, small black granules), but no cytoplasmic stain. Cytoplasmic catalase is localized at the cell periphery and in discrete blocks surrounding the nucleus. Its concentration differs between adjacent cells. Compare to electron micrograph of Fig. 5. Scale bar = 10 micrometer.

peroxisomal protein were not found. In the remainder 10% of the cells, typical peroxisomes containing the same enzymes, as well as the 43 kDa membrane protein, were seen by light- and electron microscopy; they were few in some cells. The mosaic in this patient differs from that in the first case by the regional distribution of the 'normal' cells : they are always grouped near veins (Fig. 4); the latter were recognized as portal veins in many instances, but not in all. Often, the normal cells did not form a continuous sheet, but were isolated or in groups of 2-3, near a blood vessel. By electron microscopy also, a peroxisome containing cell could be seen in contact with several others devoid of these organelles but displaying cytoplasmic regions with dark catalase reaction product (Fig. 5). The last image is similar to the cytoplasmic catalase as found in liver parenchyma of Rhesus monkey, guinea pig and sheep. However in these species the same cells possess peroxisomes as well [35, 56, 60, 63]. Peroxisomes in one tenth of the patient's parenchyma displayed an entirely normal morphology and abundant catalase reaction product (Fig 6).

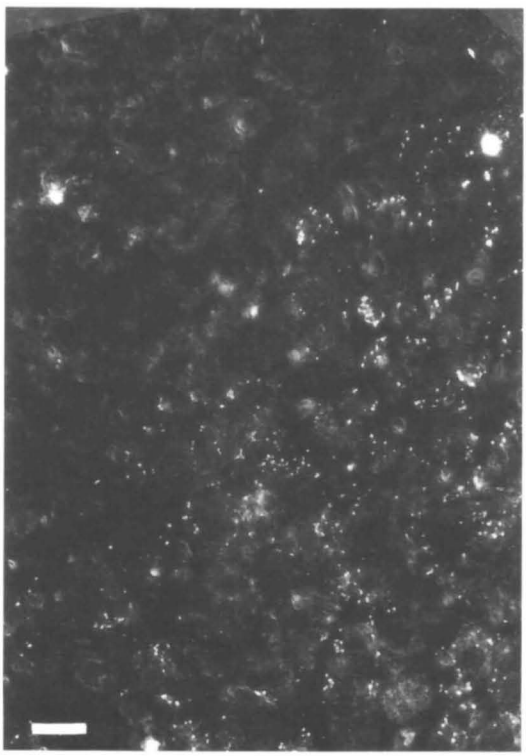

Fig. 3. Immunolocalization of alanine-glyoxylate aminotransferase in liver of patient 2. Photograph shows boundary between region of the parenchyma without peroxisomes, and a group of cells with peroxisomes, seen as numerous, bright granules. Compare to catalase localization in fig. 2. Staining for peroxisomal thiolase produced a similar picture (not shown). Darkground illumination. Scale bar = 20 micrometer.

Table 1.

| | D-Circle ($\mu$m) | | | | | | |
|---|---|---|---|---|---|---|---|
| | mean measured | corrected | maximum | 95% ° | Volume density (%) | Num. density ($\mu m^{-3}$) | Surf. density ($\mu m^{-1}$) |
| Patient 3 (RCDP) | | | | | | | |
| 8m + | 0.719 | 0.918 | 1.862 | 1.375 | 1.040 | 0.043 | 0.077 |
| 2y9m (autopsy) | 0.739 | 0.936 | 1.560 | 1.376 | 3.800 | 0.099 | 0.240 |
| Patient 4 (atypical RCDP) | | | | | | | |
| 9y⊛ | 1.119 | 1.440 | 2.767 | 2.310 | 0.422 | 0.003 | 0.018 |
| 11y | 0.934 | 1.172 | 1.897 | 1.648 | 2.350 | 0.030 | 0.120 |
| Patient 5* (IRD) | | | | | | | |
| 11y | 0.472 | 0.581 | 0.828 | 0.753 | 0.378 | 0.047 | 0.045 |
| Controls | | | | | | | |
| 7 adults | 0.525 (0.487-0.620) • | 0.643 (0.598-0.753) • | 0.940 | 0.768 (0.725-0.831) • | 1.047 (0.734-1.441) • | 0.100 (0.053-0.132) • | 0.110 (0.083-0.152) • |
| infant (6w) | 0.445 | 0.555 | 0.848 | 0.769 | 0.712 | 0.110 | 0.085 |
| infant (4m) | 0.518 | 0.640 | 1.027 | 0.848 | 1.183 | 0.128 | 0.131 |

°: mean of 5% highest values
+: from De Craemer et al., Virchows Archiv A Pathol Anat (1991) 419:523–525
⊛: from Espeel et al., Ultrastruct Pathol (1993) 17:625–637
*: from De Craemer, Ph.D. tesis, Brussels (1994)
•: minimum-maximum

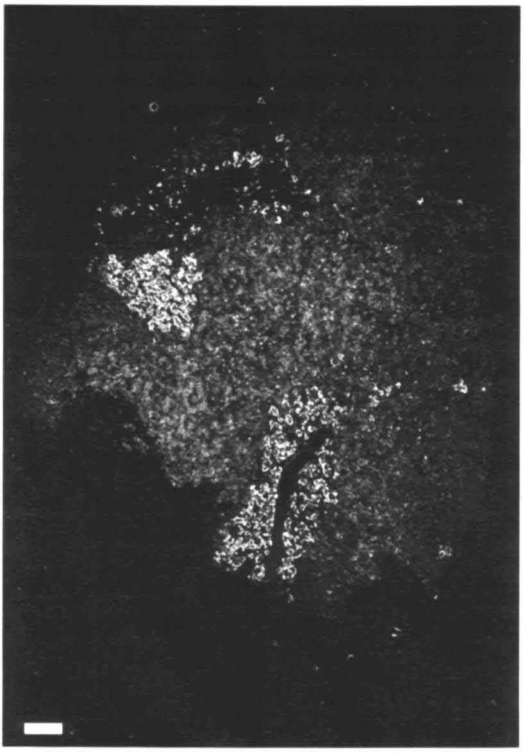

Fig. 4. Low power photograph of entire section of liver biopsy stained for catalase activity. Darkground microscopy reveals two discrete areas, one along a recognizable blood vessel, where peroxisomal staining produces strong light scatter; individual granules are not visible at this magnification. A few single cells also contain peroxisomes. In most of the section peroxisomes are absent. On the basis of such sections from all fragments of the biopsy, volume of the peroxisome-containing parenchyma was estimated at 10%. Patient No. 2. Scale bar = 100 micrometer.

The third patient is a boy with typical rhizomelic chondrodysplasia punctata (MR, patient of M.J. Zweens, Dr J.J.J. Waelkens). Peroxisomes were grossly enlarged as seen already by light microscopy [61] and confirmed by morphometry; in addition the mean number of peroxisomes in photographs taken at random was 45% of normal [19; Table I]. However this reduced number was not spread out evenly over all cells: in 2 $\mu$m sections, some cells showed few or no peroxisomes, a distribution never observed in normal livers (see Fig. 1B in [61]). By electron microscopy areas without peroxisomes were four times more frequent than in control livers. That electron microscopy of normal liver may display some cells without peroxisomes is explained by the use of ultrathin sections: organelles representing only 1% of the cellular volume are not necessarily hit in all sections.

This was found at the age of 8 months. The patient died unexpectedly from intestinal ileus at the age of 2 y 9 m; we could study another liver sample.

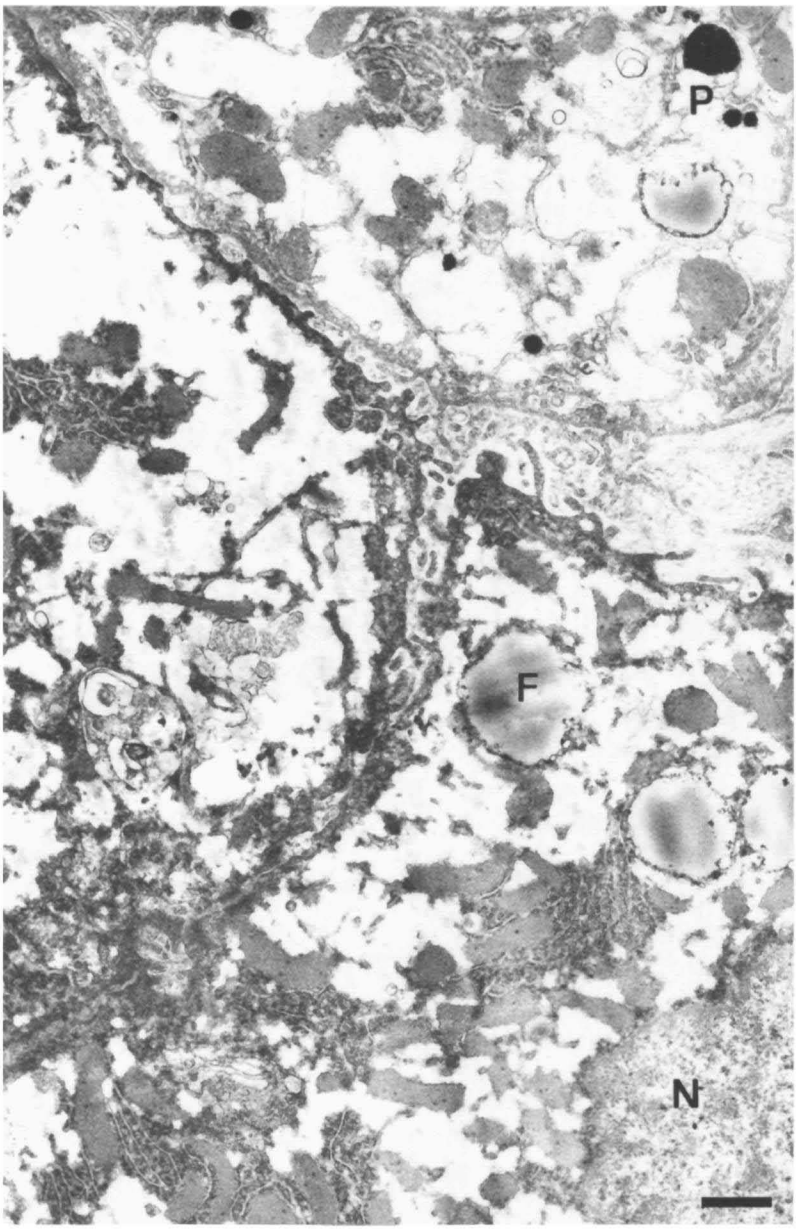


Fig. 5. Three parenchymal cells after staining for catalase activity. Upper cell has six peroxisomes of variable size (P), while two lower cells show none. However catalase reaction product has accumulated along their plasmamembranes and inside microvilli, and between the rough endoplasmic reticulum cisternae; it surrounds the bile canaliculus and Golgi apparatus. Mitochondrial matrix is less electron dense than cytoplasmic catalase reaction product. This image is similar to that of cytoplasmic catalase in the Rhesus monkey and sheep [56, 60]. Cell with peroxisomes shows less cytoplasmic density. Large 'empty' regions in cytoplasm are glycogen areas, but glycogen is unstained by the procedure used here. Fat droplets (F) are observed in two cells. N, nucleus. Patient No. 2. Scale bar = 1 micrometer.

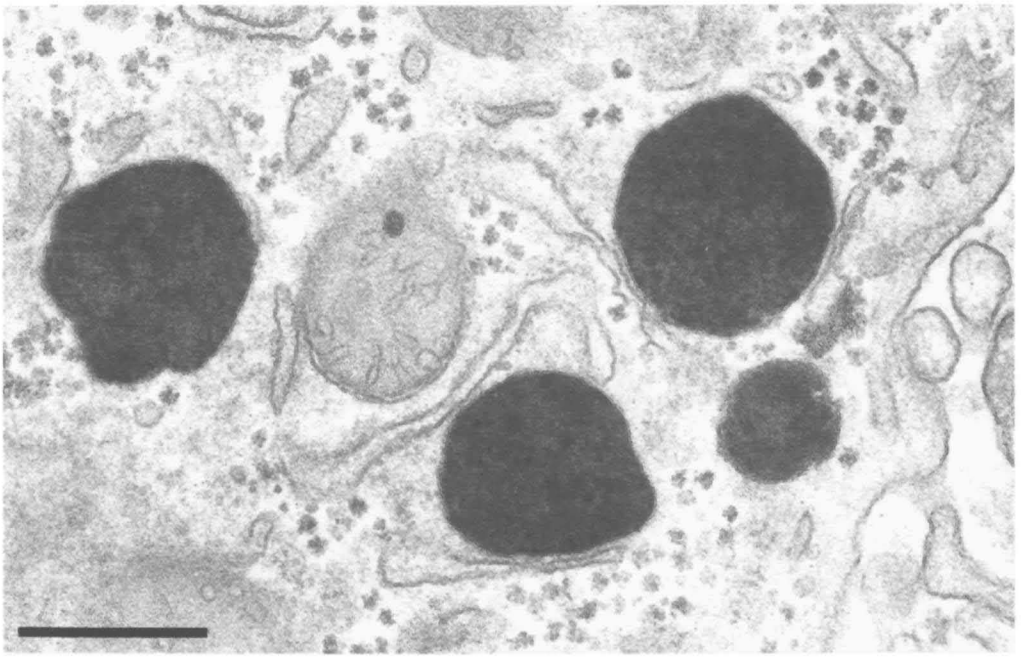

Fig. 6. Peroxisomes of patient 2 show normal shape and size and strong catalase activity. Endoplasmic reticulum is contiguous to the peroxisomal membrane, as usual. However peroxisomes are seen in 10% of the parenchyma only. Cytosol contains glycogen rosettes as in most liver biopsies. Scale bar = 0.5 micrometer.

Morphometry showed that the number of peroxisomes had increased and now was nearly normal; they were still too large and as a consequence their total volume density was three times the control value (Table I). This indicates that the mosaic observed two years earlier had changed with time.

Patient No. 4 is MD, a girl 9 years old, with non-rhizomelic CDP, under treatment by Dr Smeitink [69]. Liver pathology and immuno-cytochemistry were studied in detail by Espeel et al. [24]. The peroxisomes presented as extremely large vesicles containing four matrix enzymes except catalase, which was in the cytoplasm (photograph also in [62]). In ultrathin sections, several organelles could be seen in the same cell; however in random photographs their total number per volume Nv was only 3% of the control (Table I), proving that many cells did not show any of these organelles. Fibroblasts cultured from the skin unexpectedly showed catalase containing organelles [24].

It is important to note that this patient had a niece suffering from the same peroxisomal syndrome (reported by Pike and coworkers [51]). Although associated with a congenital rubella infection in the latter, we can assume that the disorder was genetically transmitted in both patients. So it is remarkable that Pike et al. [51] measured hepatic peroxisomes much smaller than normal ones, i.e. the opposite of our case.

As in patient No. 3, an impressive change with time was observed in patient No. 4: in a second biopsy taken 2 years after start of treatment, the number of peroxisomes had increased but was still very low: 29% of normal (Table I). Moreover, they had acquired catalase activity. Many showed membrane invaginations, a feature observed in several peroxisomal patients [57]. Similar to patient No. 3, peroxisomal volume density was increased above normal due to the large organelle size. When the distribution of peroxisomes was examined by light microscopy, they were numerous in some cells and very few in others (Fig. 7). The girl is now 11.5 y and demonstrating noticable psychomotor progress.

The last patient in our series with an abnormal distribution of peroxisomes in the liver is the oldest (JM, 11 years) of the three IRD cases originally discovered by Scotto et al. [68]. As we have shown earlier [59], numerous abnormal

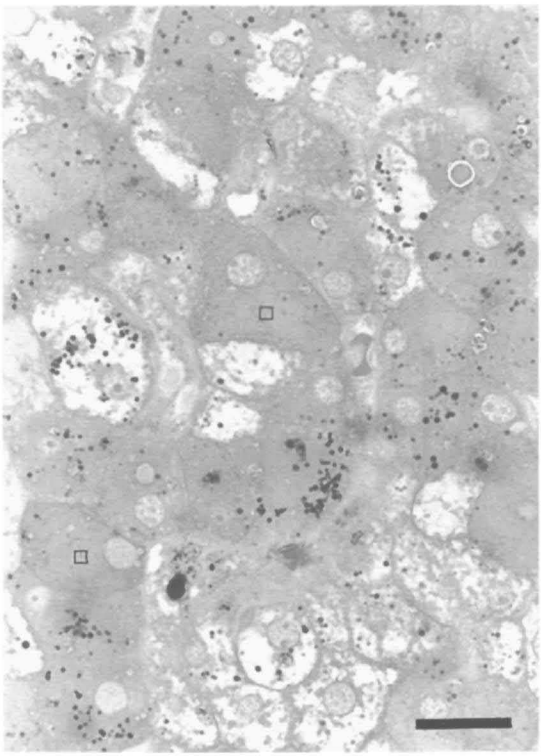

Fig. 7. Liver of patient No. 4 stained for catalase activity. Several parenchymal cells have abundant peroxisomes, other cells (squares) show only one or two granules. In contrast to patient No. 2, cells poor in peroxisomes do not form homogeneous regions but are scattered between normal looking cells. Because this is a 2 $\mu$m section, it is possible that these cells do possess more peroxisomes above or below this section. Preferential localization (polarization) of peroxisomes in hepatocytes is not expected, taken into account their distribution in normal and diseased livers. Scale bar = 10 micrometer.

microbodies were visible in some parenchymal cells: they had little catalase activity, were slightly smaller than normal, often displayed an electron lucent region and invaginations of their membrane [57, 61]. We do not know whether they contained other enzymes because in 1984, date of the biopsy, antibodies against peroxisomal proteins were not yet available for immunocytochemistry. When we published this case we assumed that the enzymes were missing because they were barely detected in immunoblots [53, 57, 61]. However, in the light of the new data from patient No. 1, these organelles might be functional peroxisomes present only in part of the parenchyma; this may be related to the clinical history of this boy who is now 19 years old. Random morphometry by De Craemer gave evidence that the mean number of these microbodies per volume Nv was only 47% of normal and the total volume 38% (Table I) confirming that the single published photograph was not average; other cells did not show any of these organelles, and such areas were three times more numerous than in control livers.

The mosaics in the liver of patients 3-5 differ from those of patients 1, 2: - by the occurrence of single cells poor in peroxisomes while in patient 1 and 2 there are homogeneous regions without any peroxisome (fig. 2 vs fig. 7); - the reciprocal presence of cytoplasmic catalase or peroxisomes in patients 1 and 2 reinforces the conclusion that peroxisomes are not fewer but totally absent.

**Disagreement between cultured fibroblasts and liver**

Many cases of such discrepancy have actually been reported, but the full significance was often not realized; in some cases cultured fibroblasts and liver were examined by different investigators and methods. One good example is the presence of peroxisomes in HPA as mentioned above. Three of our five patients also demonstrated a disagreement (No. 1, 2 and No. 4). In addition to the liver/fibroblast discrepancy, cultured cells grown from the same patient have demonstrated heterogeneity. Of both phenomena a list follows, which probably is not exhaustive: investigators may be reluctant to report such data.

*Liver vs. fibroblasts:*

- fibroblasts from recognized Zellweger patients have sedimentable catalase, thiolase [4, 29, 33], active acylCoA oxidase, dihydroxyacetone phosphate acyltransferase, the 69 kDa [77], the 70 kDa [29] and the 22 kDa peroxisomal membrane protein [33], nonspecific lipid transfer protein [72] in the same fractions; labelled granules have been observed by electron microscopy [2, 29] or by immunofluorescence [4, 29, 73, 77]. Such granules are absent in Zellweger livers. The resemblance between the particles of Zellweger cells and peroxisomes in control fibroblasts was stressed [29].
- a Zellweger variant with metabolites typical of multiple peroxisomal defects, had normal functions in fibroblasts cultured from two successive skin biopsies [67].

• fibroblasts from an IRD patient showed organelles staining strongly for catalase; the liver of the same patient had no typical peroxisomes [5].

• 20% of fibroblasts from IRD patient BOV84AD showed catalase particles and processing of acylCoA oxidase was similar to that of control fibroblasts [79]; the liver of this patient had no organelles of the peroxisome family when studied by electron microscopy and catalase cytochemistry [59].

• fibroblasts displayed no catalase latency, but the liver of the patient showed normal peroxisomes staining for catalase activity [47].

*Heterogeneity of fibroblast cultures*

• punctate fluorescence for catalase and processing of acylCoA oxidase differ between cells belonging to the same complementation group, i.e. from patients with IRD, ZS and HPA [79].

• BOV84AD fibroblasts from one of Scotto's IRD patients [68] showed absence of catalase latency [8] but in a later culture demonstrated punctate fluorescence in 20%, and normal 52 and 22 kDa forms of acylCoA oxidase [79].

• fluorescence for sterol carrier protein showed granules in some fibroblasts of an NALD patient and was diffuse in most of them [67]; acylCoA oxidase and catalase were punctate in 10% of the cells of an NALD line [73].

• in one out of seven Zellweger cell lines, part of the cells contained catalase particles; this was unexplained but the authors excluded X-linked mosaicism because the patient was a boy [66].

• Bioukar et al. [7] demonstrated the appearance of revertants, i.e. cells displaying functionally normal peroxisomes, in cultures of IRD fibroblasts, at an average frequency of 1 in $10^6$ cells.

## Implications of mosaicism and tissue heterogeneity

*For correct diagnosis*

Assays of homogenates are averages and will not detect the mosaic. Depending on the proportion of normal cells, results will be interpreted as 'peroxisomal deficiency' or as 'within the normal range.' In functional terms however, the latter case is not normal, as explained below. Normal data from cultured fibroblasts are irrelevant to liver, and vice versa. It is not yet known whether fibroblasts cultured from first trimester chorionic villi also can behave differently from liver parenchyma. For that reason it seems preferable to use uncultured trophoblast for prenatal diagnosis; this also avoids contamination by maternal cells [65].

If normal peroxisomal function observed in the fibroblasts of several peroxisome-deficient patients is not an artefact of cell culture, it demonstrates that fibroblast peroxisomes have no critical role in brain development and stabilisation; the latter appears to be more dependent on liver function, or on the oligodendrocyte peroxisomes about which we have no data in these cases. Kamei et al. [41] have reported absence of peroxisomal enzyme antigens including catalase in the cortical neurons of Zellweger patients; as in the brain of control individuals they only observed a diffuse, instead of a granular staining [37, 41], these results are difficult to interpret.

In the liver, the presence of normal peroxisomes in part of the parenchyma does not garantee normal functional capacity and is actually compatible with functional failure. This is caused by the fact that an organ is heterogeneously structured, in contrast to homogenates and cell cultures. After blood has entered the liver from the portal vein and hepatic artery, it is distributed over many lobules where it flows through sinusoids along a limited number of parenchymal cells. The blood is then collected in the central veins and returns to the heart. Each time a sinusoid is lined by parenchyma without peroxisomes, increased VLCFA and other abnormal metabolites delivered by this parenchyma will leave the liver to the brain, adrenals, a.o. No correction is possible by the normal peroxisomes localized in other parts of the same lobule or elsewhere in the liver - even when they would increase their metabolic capacity. Such an increase is not unlikely in view of animal experiments demonstrating induction of peroxisomal enzymes by fatty diets or phytanic acid [18, 57, 75]. An unexpected consequence of such increase could be that in homogenates and peroxisomal fractions, normal or even enhanced enzyme activities might be found.

**Origin of peroxisomal mosaics.**

Mosaics are well-known in mutations of mitochondrial DNA; but peroxisomes have no DNA and all their components studied so far are encoded by nuclear genes.

Two other classical explanations of a mosaic are:
- an X-linked disorder in females, because one X chromosome in each cell is inactivated at random. But 4/6 of our patients are boys.
- a somatic mutation early during liver development, such as non-disjunction of a chromosome pair during mitosis. However, two of our patients have relatives with a similar syndrome, so it is most likely that the defect was transmitted by the parents. Somatic loss of heterozygosity (reduction to homozygosity) has been demonstrated in many malignancies (breast, colon, bladder, lung, astrocytoma, renal cell carcinoma, hepatoblastoma, rhabdomyosarcoma, osteosarcoma, retino-blastoma) [28, 80]. The normal, wild type allele is lost during mitosis and the recessive gene (tumor forming) - which was inherited from one of the parents - is now expressed.

However, this is a rare event, while in two of our patients the mutated phenotype (=absence of peroxisomes) represents 90% of the liver parenchyma. This ratio might still be explained by an additional speculation, i.e. a proliferative advantage for cells without peroxisomes. A selective advantage obviously characterizes tumorcells; if so in the liver of our patient, a hepatocarcinoma might ultimately develop. DNA amplification of recessive genes is an other mechanism found in tumors [1].

In order to explain mosaicism by mitotic recombination one should assume that one of the genes coding for peroxisome assembly bears two recessive mutations, each inherited from one parent but in different loci of the gene. Recombination will result in one chromatid bearing both mutated loci, and the other bearing the wild type gene; the latter daughter cell has peroxisomes, and so will all its offspring. The frequency of such an event depends on the distance between both loci, which in turn is limited by the size of the gene: recombination within a gene of $10^6$ bp is estimated at 1 in 100 mitoses. Information on the gene(s) coding for peroxisome formation in mammalian cells is forthcoming but still fragmentary at present. According to Gärtner (personal comm.) the total DNA sequence involved in synthesis of the 70 kDa peroxisomal membrane protein [34], including the introns, is at least 100 kb. The probability of recombination within a gene of this size is still a factor 100 smaller than the frequency of the hepatic parenchymal cells possessing peroxisomes,as observed in our patients (1/10). If 'recombined' cells with peroxisomes have a proliferative advantage, this might ultimately result in clinical improvement, at least of these organ defects which are reversible. This possibility is suggested by our observation that in two patients (No. 3 & 4) the number of peroxisomes increased significantly over a period of two years.

In the first patient, mitotic recombination seems most unlikely because he has consanguinous parents; so both parents probably have the same mutation. Recently a hepatic mosaic of fumarylacetoacetase protein was demonstrated immunocytochemically by Kvittingen et al. [45] in 16 out of 19 patients with tyrosinemia type I. In four of the patients and their parents, the authors studied genomic DNA by restriction enzyme analysis. They concluded that in the enzyme-positive regions of the liver the primary mutation was reverted to wild type in one allele. This remarkable investigation was possible because a) the mutation in several families was known; b) immunostaining followed by DNA sequencing was performed on large sections through the liver after the latter was ablated for transplantation.

*Regulation of peroxisomal biogenesis by the cellular microenvironment*

Development, abundance and morphology of peroxisomes normally differ between cell types of the same species; biogenesis in fetal liver, kidney, intestinal epithelium, lung alveolar epithelium and adrenal cortex is strictly linked to specific stages [23, 42, 52]. According to Stefanini et al. [70] there are no peroxisomes visible by electron microscopy in the liver of the rat at day 13 after

fertilization. This shows that the expression of peroxisomal genes is modulated in time and by the cellular environment. Studies of cultured hepatocytes have established that expression of other liver specific genes such as albumin secretion and alpha 1-antitrypsin synthesis is dependent on the microenvironment, i.e. cell density and presence of other cell types or connective tissue components ([11, 22] reviews by Marceau [48] and L.M. Reid [54]). With respect to peroxisomes, fewer data are available. Sun et al. [71] have found that in a normal liver cell line catalase activity is approx 4% of that in liver tissue; this is accompanied by a decreased mRNA level and by higher methylation of the catalase gene DNA. In primary hepatocyte cultures catalase activity after 10 days is down to 3%, and the number of peroxisomes to 5%, of the values in freshly isolated cells [32]. We also made calculations of peroxisomal $\beta$-oxidation capacity based on published data [6, 8, 40, 46, 50], and assuming that wet liver contains 12.5% of protein. At 96 h of primary hepatocyte culture, we estimate that peroxisomal $\beta$-oxidation is decreased to 6%-15% of the activity in liver.

We hypothesize that the regulatory mechanisms for peroxisome biogenesis have mutated in our patients 1 and 2, resulting in an increased threshold for the extracellular signal (by a modification of integrins for example), or a weaker expression of this signal. As the microenvironment normally varies within the liver, the threshold will still be reached in a few regions, and normal peroxisomes will be assembled there. Patients 3 and 4 did not possess normal peroxisomes: the enzymes of plasmalogen synthesis, and 41 kDa thiolase, resp. catalase were absent in their grossly enlarged organelles; this indicates another pathogenetic mechanism. Moreover regions of liver parenchyma entirely devoid of peroxisomes were not observed. Their unequal distribution over the cells suggests an irregularity of peroxisomal division.

Skin fibroblasts obviously have a microenvironment which differs from that of liver parenchyma; in the first place they produce extracellular matrix themselves. Also they (may) express other integrins. This relates to the in vivo situation. In culture they still secrete proteoglycans. In addition inhibitory factors present in tissue can be washed out in culture. At present it is not known whether peroxisomes in cultured cells are always identical to those in the skin of the same patient, especially in peroxisomal deficiencies; the ultrastructure of cultured cells certainly is altered [62]. It does not really come as a surprise that cultured fibroblasts may behave differently from liver cells, but the practical implications are far-reaching.

## Conclusion

Future studies and diagnostic assays should take into account the existence of mosaics, and the discrepant behaviour of cultured cells. These are two reasons to examine liver biopsies, and to give priority to data from the liver. Microscopy is indispensable for the detection of mosaics. In addition earlier data need to be reinterpreted. On the other hand will the discovery of peroxisomal mosaicism

and tissue heterogeneity contribute to new insight in the determinism of peroxisomal biogenesis and its impairments in human disease; there are now indications that factors other than the peroxisomal genes may be responsible.


## Summary

Peroxisomal deficiency disorders which are genetically transmitted are assumed to be expressed in all cells and celltypes; the use of cultured fibroblasts for diagnosis and research is based on this assumption. In this review we describe five patients (in addition to a case published elsewhere (Espeel et al. [25]) with clinical, biochemical and microscopic evidence of a peroxisomal disorder. However their liver displays mosaicism, i.e. parenchymal cells with peroxisomes are adjacent to cells without, or very few, peroxisomes. Clinically the six patients presented as mild Zellweger syndrome, infantile Refsum disease, rhizomelic chondrodysplasia punctata, non-rhizomelic CDP, and one clinically unclassified. In two patients (No. 1 & 2) 8%, resp. 10% of the parenchyma possesses normal peroxisomes (containing immunoreactive thiolase, alanine-glyoxylate aminotransferase and catalase; in one patient, also acylCoA oxidase or the 43 kDa peroxisomal membrane protein). Immunoblots of patient No. 1 only showed traces of peroxisomal $\beta$-oxidation enzymes. In most of the parenchyma, catalase was localized in the cytoplasm and no organelles of the peroxisome family were seen by electron microscopy and immuno-label for the 43 kDa PMP. Patients No. 3-5 had abnormal peroxisomes unequally distributed over the parenchymal cells. As shown by morphometry on random photographs of two successive biopsies, the number of peroxisomes Nv increased over a period of two years, in two patients, from 3% to 29%, and from 45% to 90% of the number in normal livers.

This review also lists two dozen examples of disagreement between cultured fibroblasts and liver, including three of our patients, or variability of fibroblast cultures. The pathophysiology of hepatic peroxisomal mosaics is discussed; as a consequence of the vascular structure of the liver, the presence of normal peroxisomes in some regions is compatible with a biochemical deficiency in the living patient. On the other hand, if peroxisome-containing cells would have a proliferative advantage, clinical improvement might ensue over the years. As possible origin of a peroxisomal mosaic are considered : an X-linked disorder in females, a somatic mutation during liver development, somatic loss of heterozygosity, DNA amplification of recessive genes, mitotic recombination, and regulation of peroxisomal biogenesis by the cellular microenvironment. For the latter mechanism other than peroxisomal genes can be responsible. Some models are unlikely in several of our patients.

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**Note added in proof:**

A peroxisome mosaic was found in the liver of an additional peroxisomal patient, a girl of 11 years old diagnozed by Dr. M. Giros (Barcelona, Spain) described in: Roels F., T. Tijtgat, S. Beken, M. Giros, M. Espeel, B. de Prest, I. Kerckaert, T. Pampols and V. Rogiers. Peroxisome mosaics in the liver of patients and the regulation of peroxisome expression in rat hepatocyte cultures. *Ann. N.Y. Acad. Sci.*, in press.

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