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MOLECULAR ANALYSIS OF PEROXISOMAL β-OXIDATION ENZYMES IN INFANTS WITH PEROXISOMAL DISORDERS INDICATES HETEROGENEITY OF THE PRIMARY DEFECT[1]

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Immunoblot analysis of peroxisomal β-oxidation enzymes proteins was carried on liver samples from 15 patients with peroxisomal disorders in which accumulation of very long chain fatty acids was always observed in plasma.

In 11 cases including 4 cerebro-hepatorenal syndrome (CHRS), 4 neonatal adrenoleukodystrophy (NALD) and 3 infantile Refsum's disease, the liver peroxisomes could not be detected by electron microscopy. Immunoblot analysis revealed the absence, or presence in weak amounts, of the 72-kDa subunit of acyl-CoA oxidase, and the complete absence of the 52-kDa and 21-kDa subunits which are processed from the 72-kDa. The bifunctional protein (78-kDa) was absent or very reduced, as was the mature form of peroxisomal 3-ketoacyl-CoA thiolase (41-kDa). Multiple defects of peroxisomal β-oxidation enzymes may be caused by an absence of synthesis or an inability to import proteins into peroxisomes in these patients.

One patient, diagnosed as NADL, had no detectable liver peroxisomes but the presence, in normal amounts, of the three peroxisomal β-oxidation enzyme proteins suggests that the transport of these enzymes into "peroxisomal ghosts" was still intact.

The last 3 patients, clinically diagnosed as NALD, had normal liver peroxisomes. One patient had an isolated deficiency of the bifunctional protein and the 2 others had normal amounts of the 3 peroxisomal β-oxidation enzymes, as shown by immunoblotting. This suggests that import and translocation of some peroxisomal proteins had occurred and that a mechanism is therefore required to explain the defect in these patients.

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Peroxisomal disorders are a group of metabolic diseases in which one or several peroxisomal enzymes are deficient (1-3).

With the exceptions of primary hyperoxaliuria type 1, the adult form of Refsum's disease and the rhizomelic chondrodysplasia punctata, the oxidation of very long chain fatty acids (VLCFA) is always impaired in these disorders. Hence, the disturbance of peroxisomal β-oxidation results in a accumulation of VLCFA in plasma and tissues (4). This may occur either singly, or in association with other peroxisomal metabolic defects.

VLCFA oxidation deficiency associated with other biochemical abnormalities was first established in the Zellweger cerebrohepatorenal syndrome (CHRS) and then extended to other peroxisomal entities, i.e. neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD). In addition to the accumulation of VLCFA, these patients have an impaired synthesis of plasmalogen and bile acids, and an accumulation of pipecolic acid, dicarboxylic acids and phytanic acid (2).

These multiple enzyme defects are considered to be caused secondarily by the lack or paucity of peroxisomes (4-6).

The initial step of peroxisomal β-oxidation, i.e. the activation of VLCFA to their CoA-esters derivatives is carried by a specific VLCFA CoA synthetase. The remaining steps involve three enzyme proteins, a fatty acyl-CoA oxidase (FAOx), a bifunctional protein (BFP) with enoyl-CoA hydratase and 3-hydroxy acyl-CoA dehydrogenase activities, and a 3-ketoacyl-CoA thiolase (thiolase) (7, 8). Previous studies showed that the accumulation of very long chain fatty acids in patients with CHRS, NADL or IRD (9-12) can be attributed to the lack of the final 3 enzyme proteins of peroxisomal β-oxidation (13-16). Pulse chase experiments (17) and "in vitro" translation studies (18), suggested that the lack of FAOx, BPF and thiolase is due to a rapid degradation of these enzyme proteins in the absence of peroxisomes rather than to the lack of their synthesis.

We have carried immunoblot analysis with liver samples and compared pattern of peroxisomal β-oxidation enzymes deficiencies in 4 patients with accumulation of VLCFA to those of patients with classical CHRS, NALD or infantile Refsum's disease.

Materials and Methods

Liver samples were obtained from three fetuses and twelve infants with peroxisomal disorders. Livers from fetuses were obtained after abortions following antenatal diagnosis. The cytochemical demonstration of catalase activity in liver samples was performed according to the procedure of Roels and Goldfisher (19). The deficiency of peroxisomal β-oxidation was ascertained in the 15 patients by the demonstration of

Table 1

VLCFA levels in plasma [a]

	Number patients	C24:0/C22:0	C26:0/C22:0
Control (n = 30)		0.68±0.20	0.011±0.007
Zellweger syndrome	2	1.78	0.738
	3	1.72 *	0.712 *
	11	1.91 *	0.723 *
	12	1.47	0.413
Neonatal adrenoleukodystrophy	4	1.32	0.182
	5	1.67	0.208
	6	1.21	0.232
	7	1.18	0.402
Infantile Refsum's disease	8	2.14	0.630
	9	1.42 *	0.192 *
	13	1.10 *	0.204 *
PDD	15	1.47	0.070
	16	1.32	0.120
	17	1.09	0.065
	18	1.20	0.082

a : Fatty acid ratios of VLCFA was determined by capillary gas liquid chromatographic-mass spectrometry according to the method of P. Aubourg et al. (20).

PPD : peroxisome deficiency disorders (clinically diagnosed as NADL)

* : refer to values found in index cases of studied fetuses.

accumulation of VLCFA in plasma (Table 1). In 7 cases (data not shown) this defect was substantiated by the study of oxidation of $|^{14}C|$ lignoceric acid and the accumulation of VLCFA in fibroblasts.

Furthermore, distinction between CHRS, NALD and IRD was performed in 11 cases according to clinical, biochemical and morphological criteria (21, 22). Thus, infant cases included 2 CHRS, 4 NALD and 2 IRD. Two fetuses had CHRS and one IRD.

Control livers were obtained from two adults who were in irreversible coma, from a 12 years old child operated for portal cavernoma and from a 14 weeks old normal fetus.

Tissue samples were stored at - 80° C, and allowed to thaw in ice-cold buffer according to Chen et al. (15). They were then homogenized in a Potter-Elvehjern homogenizer. Homogenates were sonicated and cleared by brief centrifugation. Samples were stored at - 20° C. Aliquots containing approximately 4-5 µg protein were subjected to polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose filters according to the method of Burnette (23).

Antibodies were raised against purified rat liver FAOx, BFP and thiolase (24-28). These antibodies had previously been shown to cross react with the corresponding enzymes of human liver (13). Filters were first blocked with 4 % bovin serum albumin at room temperature for 30

minutes followed by incubation overnight with antibody against each of the enzyme proteins. After washing, the filters were incubated for 2 hours with $|^{125}I|$ labeled protein A (apprimately $6x10^5$ cpm/ml). Autoradiography was carried out on Kodak X-Omat AR film with or without intensifying screens at - 80° C or at room temperature.

Results

In normal mature livers (fig. 1 lanes 1, 10 and 19), the usual banding pattern of to the three enzyme proteins studies was observed. FAOx was composed of three polypeptides A, B and C whose molecular weights were 72-, 52- and 21-kDa, respectively (fig. 1A, arrows). A proteolytic cleavage is thought to generate polypeptides B and C from A in the cell. BFP corresponded to a unique 78-kDa protein (fig. 1B, arrow). Thiolase was essentially seen as a 41-kDa protein (fig. 1C, arrow) with a faint signal of the 44-kDa precursor form.

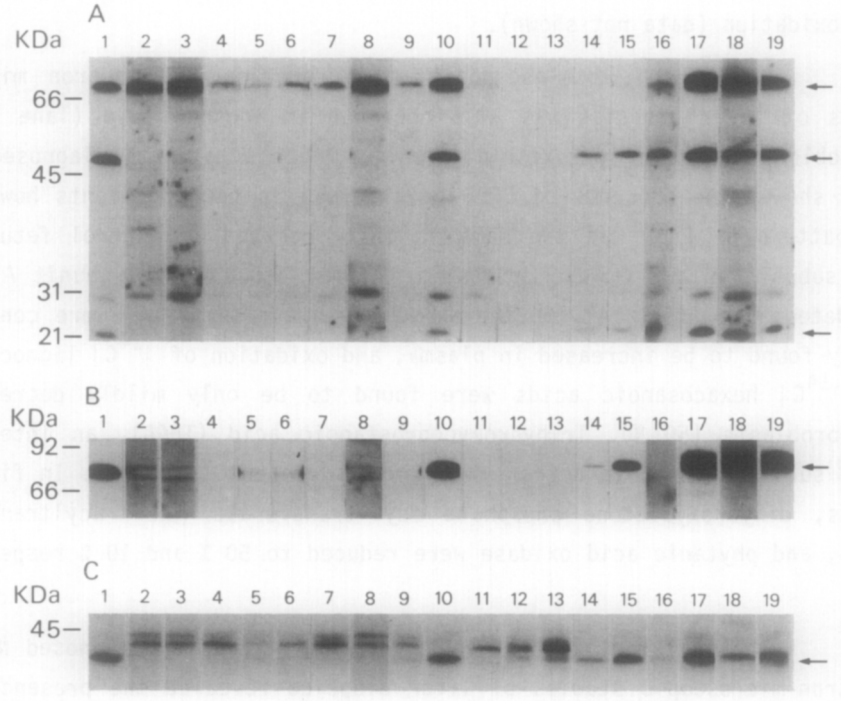

Figure 1:
Immunoblots with antibodies raised against (A) fatty acyl-CoA oxidase (FAOx), (B) bifunctional protein (BFP), (C) 3-ketoacyl-CoA thiolase (thiolase). Autoradiography was carried out for 48 hours at - 80° C with 2 screens for (A) and (B), and during 17 hours at room temperature without a screen for (C). Lanes 1, 10 and 19 refer to normal livers, and lane 14 to a 14 weeks old fetal liver. Lanes 2 and 3 : classical cerebro-hepatorenal syndrome (CHRS), lanes 4 to 7 : neonatal adrenoleukodystrophy (NALD), lanes 8 and 9 : infantile Refsum's disease (IRD), lanes 11 and 12 : fetal liver of CHRS, lane 13 : fetal liver of IRD, lanes 15 to 18 : patients with peroxisome deficiency disorders (PDD) (clinically diagnosed as NALD).

Immunoblots of normal fetal liver (fig. 1, lane 14) showed the same protein pattern, except for the 72-kDa FAOx protein which was not detected.

In eleven cases including 8 infants (2 CHRS, 4 NALD and 2 IRD) and 3 fetuses (2 CHRS and 1 IRD) immunoblots of peroxisomal β-oxidation enzyme proteins gave identical banding patterns. Neither subunit B or C of FAOx was detected in any patients studied while subunit A was faintly detected in infants (fig. 1A, lanes 2 to 9) but not in fetus livers (fig. 1A, lanes 11 to 13). Bifunctional protein (fig. 1B) was markedly reduced or not detectable. The 41-kDa polypeptide of peroxisomal thiolase (fig. 1C) was either barely detected or absent, which contrasted with the increased intensity of the band of the 44-kDa precursor and the appearance of a 43-kDa intermediate form in high amount.
In these eleven cases, liver peroxisomes were not observed by electron microscopy and the metabolic peroxisomal impairment extended to bile acids and plasmalogen synthesis, pipecolic acid degradation and phytanic acid oxidation (data not shown).

Absence of catalase-positive microbodies in electron micrographs of liver biopsies was also observed in another case (lane 15). Immunoblot studies of peroxisomal enzymes in this patient, diagnosed as NALD, showed the presence of BFP and thiolase in normal amounts however the pattern of FAOx was the same as that observed in control fetuses, i.e. subunits B and C were present in normal amounts but subunit A was not detectable (fig. 1A). Furthermore, in this case, VLCFA were consistently found to be increased in plasma, and oxidation of $|^{14}C|$ lignoceric and $|^{14}C|$ hexacosanoic acids were found to be only mildly decreased in fibroblasts (50 %). Trihydroxycoprostanoic acid (THCA), an intermediate substance of bile acids synthesis was present in plasma. In fibroblasts, dihydroxyacetone phosphate acyl-transferase (DHAP acyltransferase), and phytanic acid oxidase were reduced to 50 % and 10 % respectively.

In the three last patients, who had clinically diagnosed NALD, electron-microscopic studies of liver biopsies revealed the presence of peroxisomes with normal size.

In one patient (lane 16), immunoblots of peroxisomal β-oxidation enzyme proteins showed the complete absence of BFP (fig. 1B) with the presence of the two other enzymes, but in lesser amounts for the thiolase (fig. 1C). The level of VLCFA was increased in plasma but normal in fibroblasts. Furthermore, phytanic acid was slightly increased in plasma and study of phytanic acid oxidase revealed normal activity in fibro-

blasts. In addition, DHAP acyltransferase activity was only mildly decreased in fibroblasts (50 %).

The three β-oxidation enzyme proteins were present in normal quantities in the last two patients (lanes 17 and 18). The level of VLCFA was increased in plasma in contrast with normal levels of THCA or phytanic acid. However, analysis of enzyme activities could not be performed, because of the lack of fibroblast cultures of these patients.

Discussion

Immunoblot analysis revealed that the three peroxisomal β-oxidation enzymes were all deficient in our patients with classical Zellweger syndrome, as reported previously (13, 17). However, complementation studies have identified at least 4 different groups within peroxisomal disorders characterized by the virtual absence of peroxisomes (29). In the first patients, we did not observe any significant difference in the pattern of these enzyme defects between classical Zellweger and patients with NALD or IRD.

In the three fetuses with CHRS or IRD, the three subunits of FAOx were absent whereas the subunit A was present in post-natal liver of the 4 cases presenting identical peroxisomal disorders. This difference can be explained by the absence or a lower rate of synthesis of the subunit A in normal fetal liver at an early gestational age. Our results suggest that subunit A of FAOx is normally synthesized in patients with CHRS, NALD or IRD, but that the processing of the subunit A to B and C is disturbed. This hypothesis has been substantiated by pulse labelling and chase experiments in CHRS or IRD fibroblasts (17, 30). These studies have shown a normal synthesis of subunit A, and the absence of processing to subunits B and C.

In some of these eleven patients, BFP was present but in faint amounts suggesting that this enzyme is very unstable in absence of normal peroxisomal structure.

Human peroxisomal 3-ketoacyl-CoA thiolase is considered to be first synthesized in the 44-kDa precursor form. Then a proteolytic cleavage processed to the 41-kDa mature form. In our patients with CHRS, NALD or IRD, the 44-kDa precursor was present but was barely or not at all processed to the mature form. The significance of the 43-kDa intermediate form is largely unknown.

So, in this group of peroxisomal disorders with generalized impairment of peroxisomal functions, the presence of the three enzymes of peroxisomal

β-oxidation, even in abnormal forms or amounts, demonstrates that they are normally synthesized in CHRS and IRD as well as in NALD patients. The absence of a targeting peroxisome structure would explain the disturbed processing of FAOx and thiolase, and the rapid degradation of these proteins in the cytosol.

In one case (lane 15), liver peroxisomes were absent, although FAOx, BFP and thiolase were present in the mature and processed forms. We hypothesize that, contrarily to catalase, these enzyme proteins were targeted to membrane structures where they have some enzymatic activities. The absence of recognizable peroxisomes by the DAB reaction does not always exclude the existence of peroxisomal membrane structure (31, 32).

Santos et al. (32) have recently provided evidence that in CHRS, the 22-kDa membrane protein (as well as other membrane proteins) is located in unusual, empty membrane structures called "peroxisomal ghosts" with a larger size than that of normal peroxisomes.

One of the case (lane 16) with peroxisomes may be related to the unique deficiency of the BFP activity. Such a deficiency is concordant with the increased level of VLCFA in plasma and with a perturbed peroxisomal β-oxidation. An impairment in only one peroxisomal function was observed in pseudo Zellweger syndrome (thiolase deficiency) (33) and pseudo neonatal adrenoleukodystrophy (acyl-CoA oxidase deficiency) (34). However, the reduced quantity of the 41-kDa thiolase protein, a mild impairment of plasmalogen synthesis and an increased level of phytanic acid in plasma could question the reality of the single defect of the BFP.

However, immunoblot studies revealed normal amounts of FAOx, BFP and thiolase in two patients with liver peroxisomes (lanes 17 and 18). Both clinically and biochemically, these two examples resemble to the peroxisomal entity recently reported by Naidu et al. (35). A more precise definition of the enzyme defect is required in these 2 cases but it seems likely that at least one of the 3 peroxisomal β-oxidation proteins is enzymatically inactive due to either structural or environmental reasons.

Immunoblot studies of peroxisomal β-oxidation enzymes reinforces the concept that the phenotypic and genotypic spectrum of peroxisomal disorders is far from been clarified. Recently, Suzuki et al. reported one patient with a clinical presentation undistiguinshable from classical Zellweger syndrome. Peroxisomes were present in liver but the 3 peroxisomal β-oxidation enzyme proteins were found to be absent as shown by immunoblotting (30, 36). Altogether, these observations suggest that

the primary defect of peroxisomal disorders is heterogenous and that deficiency of enzymes of peroxisomal β-oxidation may be caused either by absence of synthesis, abnormality of transport, or by absence of a factor that stabilizes these enzymes into peroxisomes.

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References

1. Moser, H.W., and Goldfischer, S.L. . (1985) Hospital Practice, september 15, 61-70.

2. Schutgens, R.B.H., Heymans, H.S.A. , Wanders, R.J.A., van den Bosch, H., and Tager, J.M. (1986) J. Pediatr. 144, 430-440.

3. Goldfischer, S.L. (1988) In Biology and Pathobiology. Second Edition. (I.M. Arias, W.B. Jakoby, H. Popper, D. Schachter and D.A. Shafritz, Eds). Chapter 14 pp. 255-267. Raven Press, Ltd, New York.

4. Goldfischer, S., Collins, J., Rapin, I., Coltoff-Schiller, B., Chang, C.H., Nigro, M., Black, V.H., Javitt, N.B., Moser, H.W., and Lazarow, P.B. (1985) Science 227, 67-70.

5. Goldfischer, S., Moore, C.L., Johnson, A.B., Spiro, A.J., Valsamis, M.P., Wisniewski, H.M., Ritch, R.H., Norton, W.T., Rapin, I, and Gartner, L.M. (1973) Science 182, 62-64.

6. Roels, F., Cornelis, A., Poll-The, B.T., Aubourg, P., Ogier, H., Scotto, J.M, and Saudubray, J.M.. (1986) Am. J. Med. Genet. 25, 257-271.

7. Hashimoto, T. (1982) Ann. N.Y. Acad. Sci. 386, 5-12.

8. Osumi, T., Hijikata, M., Ishii, N., Miyazawa, S., and Hashimoto, T. (1987) In Peroxisomes in Biology and Medicine (H.D. Fahimi and H. Sies, Eds), pp. 105-114. Springer-Verlag, Berlin, Heidelberg.

9. Brown, F.R., Mc Adams, A.J., Cummins, J.W., Konkol, R., Singh, I., Moser, A.B., and Moser, H.W. (1982) Johns Hopkins Med. J. 151, 344-361.

10. Moser, A.B., Singh, I., Brown, F.R., Solish, G.I., Kelley, R.I., Benke, P.J., Burton, B.K., and Moser, H.W. (1984) N. Engl. J. Med. 310, 1141-1146.

11. Vamecq, J., Draye, J.P., Van Hoof, F., Misson, J.P., Evrard, P., Verellen, G., Eyssen, H.J., Van Eldere, J., Schutgens, R.B.H., Wanders, R.J.A., Roels, F., and Goldfischer, S.L.. (1986) Am. J. Pathol. 125, 524-535.

12. Poll-The, B.T., Saudubray, J.M., Ogier, H., Schutgens, R.B.H., Wanders, R.J.A., Schrakamp, G., Van Den Bosch, H., Trijbels, J.M.F., Poulos, A., Moser, H.W., Van Eldere J., and Eyssen, H.J.. (1986) J. Inher. Metab. Dis. 9, 169-174.

13. Tager, J.M., Ten Harmsen Van Der Beek, W.A., Wanders, R.J.A., Hashimoto, T., Heymans, H.S.A., Van Den Bosch, H., Schutgens, R.B.H., and Schram, A.W. (1985) Biochem. Biophys. Res. Commun. 126, 1269-1275.

14. Suzuki, Y., Orii, T., Mori, M., Tatibana, M., and Hashimoto, T. (1986) Clin. Chim. Acta 156, 191-196.

15. Chen, W.W., Watkins, P.A., Osumi, T., Hashimoto, T., and Moser, H.W. (1987) Proc. Natl. Acad. Sci. 84, 1425-1428.

16. Shimozawa, N., Suzuki, Y., Orii, T., and Hashimoto, T. (1988) Prenatal Diagnosis 8, 287-290.

17. Schram, A.W., Strijland, A., Hashimoto, T., Wanders, R.J.A., Schutgens, R.B.H., Van Den Bosch, H., and Tager, J.M. (1986) Proc. Natl. Acad. Sci. 83, 6156-6158.

18. Suzuki, Y., Orii, T., and Hashimoto, T. (1986) J. Inher. Metab. Dis. 3, 292-296.

19. Roels, F., and Goldfisher, S. (1979) J. Histochem. Cytochem. 27, 1471-1477.

20. Aubourg, P., Bougnères, P.F., and Rocchiccioli, F. (1985) J. Lipid Res. 26, 263-267.

21. Kelley, R.I., Datta, N.S., Dobyns, W.B., Hajra, A.K., Moser, A.B., Noetzel, M.J., Zackai, E.H., and Moser, H.W. (1986) Am. J. Med. Genet. 23, 869-901.

22. Monnens, L., and Heymans, H. (1987) J. Inher. Metab. Dis. 10 suppl. 1 : 23-32.

23. Burnette, W.N. (1981) Analytical Biochem. 112, 195-203.

24. Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 87, 1735-1746.

25. Furuta, S., Miyazawa, S., Osumi, T., Hashimoto, T., and Ui, N. (1980) J. Biochem. 88, 1059-1070.

26. Osumi, T., and Hashimoto, T. (1979) Biochem. Biophys. Res. Commun. 89, 580-584.

27. Miyazawa, S., Furuta, S., Osumi, T., Hashimoto, T., and Ui, N. (1981) J. Biochem. 90, 511-519.

28. Bronfman, M., Inestrosa, N.C., Nervi, F.O., and Leighton, F. (1984) Biochem. J. 224, 709-720.

29. Tager, J.M., Westerveld, A., Strigland, A., Schram, A.W., Schutgens, R.B.H., Van den Bosch, M., and Wanders, R.J.A. (1987) In Peroxisomes in Biology and Medecine (H.D. Fahimi and H. Sies, Eds) pp. 353-357, Springer Verlag, Berlin, Heidelberg.

30. Suzuki, I., Shimozawa, N., Orii, T., Igarashi, N., Koni, N., and Hashimoto, T. (1988) Clin. Chim. Acta 172, 65-76.

31. Santos, M.J., Imanaka, T., Shio, H., Small, G.M., and Lazarow, P.B. (1988) Science 239, 1536-1538.

32. Santos, M.J., Imanaka, T., Shio, H., and Lazarow, P.B. (1988) J. Biol. Chem. 263, 10502-10509.

33. Schram, A.W., Goldfischer, S., van Roermund, C.W.T., Brouwer-Kelder, E.M., Collins, J., Hashimoto, T., Heymans, H.S.A., van den Bosch, H., Schutgens, R.B.H., Tager, J.M., and Wanders, R.J.A. (1987) Proc. Natl. Acad. Sci. 84, 2494-2496.

34. Poll-The, B.T., Roels, F., Ogier, H., Scotto, J., Vamecq, J., Schutgens, R.B.H., Wanders, R.J.A., van Roermund, C.W.T., van Wijland, M.J.A., Schram, A.W., Tager, J.M., and Saudubray, J.M. (1988) Am. J. Hum. Genet. 42, 422-434.

35. Naidu, S., Hoefler, G., Watkins, P.A., Chen, W.E., Moser, A.B., Hoefler, S., Rance, N.E., Powers, J.M., Beard, M., Green, W.R., Hashimoto, T., and Moser, H.W.. (1988) Neurology 38, 1100-1107.

36. Suzuki, Y., Shimozawa, N., Orii, T., Igarashi, N., Kono, N., Matsui, A., Inone, Y., Yokota, S. and Mashimoto, T. (1988) J. Pediatr. 113, 841-845.