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The PNPLA family of enzymes: characterisation and biological role

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This paper brings a brief review of the human patatin-like phospholipase domain-containing protein (PNPLA) family. Even though it consists of only nine members, their physiological roles and mechanisms of their catalytic activity are not fully understood. However, the results of a number of knock-out and gain- or loss-of-function research models suggest that these enzymes have an important role in maintaining the homeostasis and integrity of organelle membranes, in cell growth, signalling, cell death, and the metabolism of lipids such as triacylglycerol, phospholipids, ceramides, and retinyl esters. Research has also revealed a connection between PNPLA family member mutations or irregular catalytic activity and the development of various diseases. Here we summarise important findings published so far and discuss their structure, localisation in the cell, distribution in the tissues, specificity for substrates, and their potential physiological role, especially in view of their potential as drug targets.

KEY WORDS: catalytic dyad; lipid droplets; patatin; phospholipases; serine hydrolases

Serine hydrolases are a large class of enzymes consisting of more than 200 serine proteases, extracellular and intracellular lipases, cholinesterases and certain phospholipases, amidases, and peptidases (1) that participate in numerous biological processes such as energy metabolism, inflammation, neurotransmission, oxidation stress, and apoptosis (1, 2–5).

This class includes a family of nine enzymes called the patatin-like phospholipase domain-containing proteins (PNPLA) after the patatin-like protein structure of the catalytic domain they all share (4, 5). This structure was initially discovered in patatin (6–8), a glycoprotein highly abundant in potato tubers (Figure 1) (6). The active site of all PNPLA enzymes is situated in the patatin-like domain (α/β fold structure), where the catalytic dyad serine-aspartic acid and the oxyanion hole are positioned (4–8). Catalytic serine is a part of the Gly-X-Ser-X-Gly (X stands for any amino acid) motif, and aspartic acid is a part of the Asp-Gly-Ala/Gly motif found in most lipases and all enzymes containing the patatin-like domain (1, 4, 5). This active serine binds a substrate covalently through its functional nucleophilic hydroxyl group, while the aspartic acid plays a role of both general acid and general base, helping serine to bind substrate and regenerate. In turn, the oxyanion hole, located in close proximity to the active dyad, stabilises the transition state of the substrate hydrolysis reaction (1–9).

The PNPLA family is also known as Ca^{2+}-independent phospholipases A_2 ($iPLA_2$s), as they do not require Ca^{2+} for their activity or translocation (4, 10–14). Even though PNPLA enzymes are classified as phospholipases A_2, certain family members can also act as lysophospholipases and transacylases. In addition, some *in vitro* studies have shown that some family members can hydrolyse *sn*-1 and/or *sn*-2 substituents (12, 13), while some prefer triacylglycerol, cholesteryl, and retinyl esters as substrates (4) (Figure 2).

PNPLA enzymes are expressed in many mammalian species, and their orthologues are found in other eukaryotes such as amoebae, nematodes, yeasts, insects, and other vertebrates (10, 13, 14), but this review mainly focuses on PNPLA enzymes in humans. Their physiological role is yet to be clarified, but what we know is that some mutations, gene polymorphisms, and irregular activity of certain family members are associated with various conditions and diseases (4, 5). For example, *PNPLA2* mutations can result in the accumulation of triacylglycerol in almost every tissue of the body, *PNPLA3* mutations are associated with several liver diseases (12–15), and *PNPLA6* and *PNPLA9* mutations and irregular activity of encoded enzymes are associated with neurological disorders and neurodegeneration (16, 17).

PNPLA ENZYME CLASSIFICATION

Judging by similar domain structure (predicted for most by homology modelling) and substrate preferences, most PNPLA enzymes belong to either the adiponutrin (ADPN) group (PNPLA1–5) or the neuropathy target esterase (NTE) group (PNPLA6 and 7). The two remaining enzymes, PNPLA8 and PNPLA9 belong to neither group (4) due to the specific characteristics that will be addressed later in the text.

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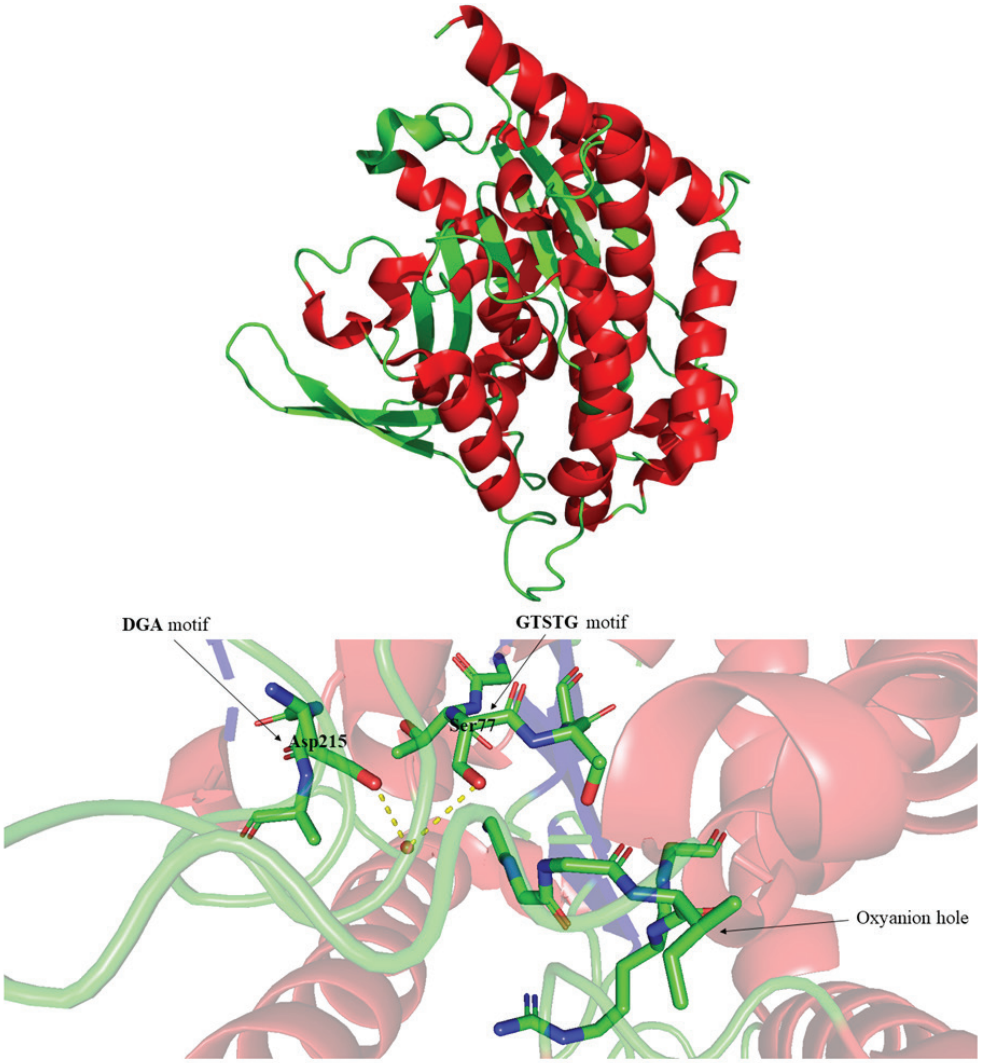

Figure 1 3D illustration of the patatin structure (PDB: 1OXW) (created with PyMol). α-helices are coloured red and β-helices green (top). The crystal structure of patatin was solved in 2003 and its core consists of the α/β fold with about three layers of the α/β/α sandwiches, in which the β-sheet is sandwiched between two α-helices front and back. In the active site (bottom), patatin contains the catalytic dyad serine-aspartic acid. Catalytic serine is situated at a nucleophilic elbow following a β-sheet and preceding an α-helix. Serine is part of the classical lipase Gly-Thr-Ser-Thr-Gly motif, and aspartic acid is part of the Asp-Gly-Ala motif. Close to the active site is an oxyanion hole characterised by the Gly-Gly-Gly-Arg motif, whose function is to stabilise the transition state. Yellow dashed lines represent hydrogen bonds between serine and water and aspartic acid and water (molecule of water is marked with a red sphere)

PNPLA1 (EC 2.3.1.296)

PNPLA1 consists of 532 amino acids composed of the *N*-terminal domain with the active site and the C-terminal domain. The patatin-like domain (residues 16–185) is located in the *N*-terminal domain and accommodates the catalytic dyad Ser53-Asp172 (5, 18, 19). Serine and aspartic acid are part of the lipase motifs, namely GTSAG (residues 51–55) and DGG (residues 172–174), respectively (5, 18, 19). In cells, PNPLA1 is located in the cytoplasm as a free enzyme but can bind to lipid droplets with its proline-rich sequence located in the C-terminal domain and positioned in amino acid residues 326–421 (20–22). Lipid droplets, also known as adiposomes, are dynamic organelles containing triacylglycerol and cholesteryl esters enclosed by a phospholipid membrane situated in the cytosol of almost every type of cell in the organism (23). *In vitro* studies show that the comparative gene identification-58 (CGI-58) enzyme, also known as the α/β hydrolase domain-containing protein 5 (ABHD5), recruits PNPLA1 to lipid droplets and enhances its enzymatic activity (21, 24), but the exact mechanism of their interaction is not fully understood. In addition, deletion of the C-terminal domain disables PNPLA1 binding to lipid droplets (18, 19).

The mRNA of *PNPLA1* is highly expressed in the granular layer of epidermis (4, 5, 18, 25), and some *in vitro* and *in vivo* studies (25–30) show that PNPLA1 expression increases during keratinocyte differentiation, as PNPLA1 plays a crucial role in the biosynthesis of ω-O-acylceramide, a lipid essential for the development and normal function of the skin, that is, for the formation of the skin permeability barrier.

However, mutations in *PNPLA1* at both C- and *N*-terminal domains are associated with the development of autosomal recessive congenital ichthyosis (ARCI) (19, 25) and cause an abnormal lipid

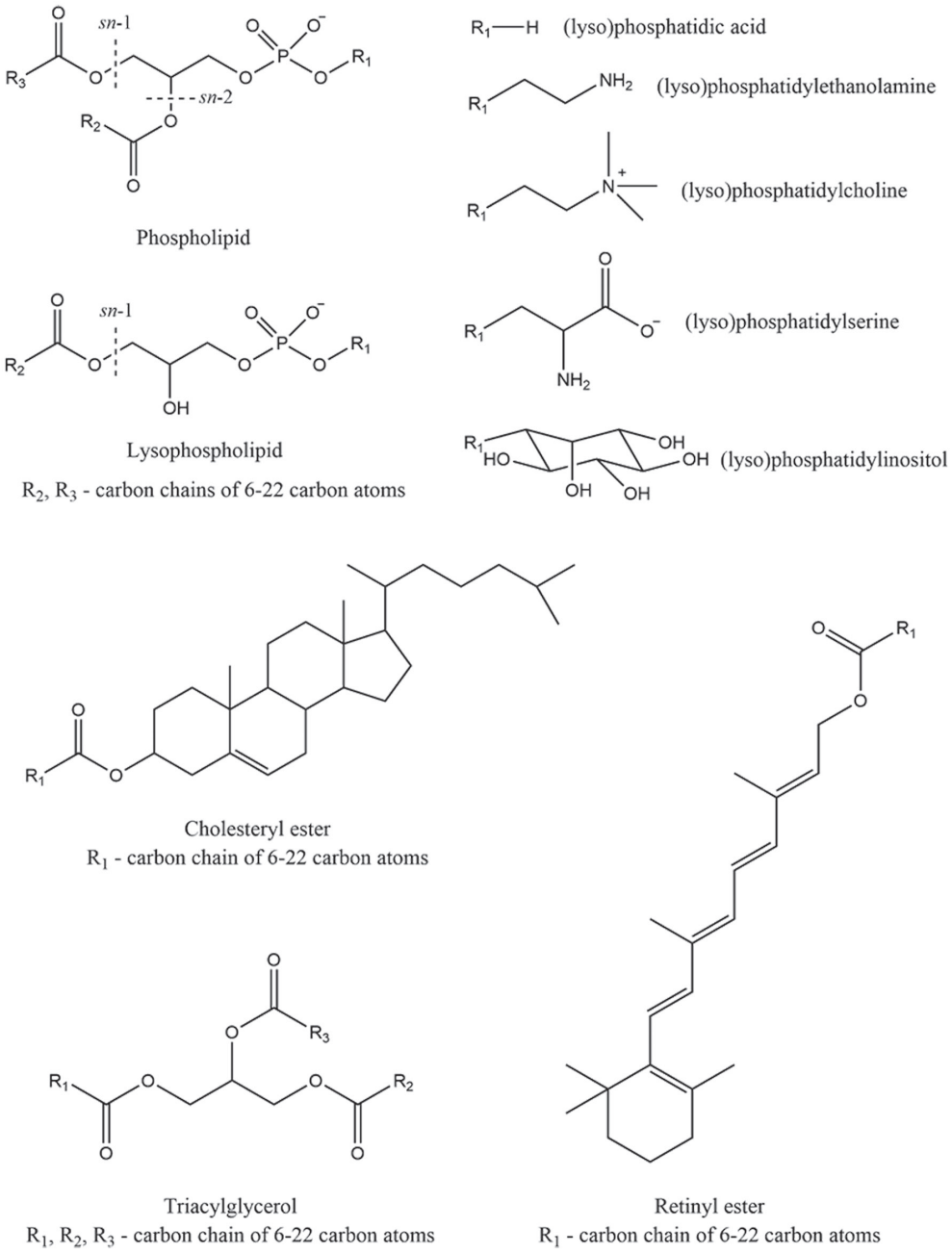

Figure 2 Substrates of the PNPLA enzymes. Some PNPLAs can hydrolyse *sn*-1, *sn*-2 or ester bonds on both positions in phospholipids and lysophospholipids (R_1 represents examples of modified phosphate groups; R_2, R_3 represent alkyl groups which can contain 6–22 carbon atoms and one or more double bonds). Certain members can hydrolyse ester bonds of triacylglycerol, cholesteryl ester, and retinyl ester

droplet accumulation in fibroblast cells and impair ω-O-acylceramide biosynthesis (22). Consequently, individuals affected by ARCI usually have thick, dry, and scaly skin (25).

PNPLA2 (EC 3.1.1.3)

PNPLA2, also known as adipose triglyceride lipase (ATGL) or desnutrin, contains 504 amino acids and is important for energy metabolism (4, 5). It is structured around two domains: *N*-terminal, with the active site, and C-terminal. The *N*-terminal domain contains the patatin domain in the amino acid residue region 10–179 (4, 31), which has a similar position and contains the catalytic dyad Ser47-Asp166 (with the classical lipase motifs GASAG and DGG, respectively, just like PNPLA1). PNPLA2 is present in the cytosol or bound to lipid droplets with the hydrophobic sequence of 40 amino acid residues located on the C-terminal domain (20, 32, 33). *In vitro* and *in vivo* studies (31, 34–38) show that PNPLA2 is expressed in almost every tissue, including the cardiac muscle, skeletal muscle, testis, and liver, but most dominantly in the white and brown adipose

tissue, in which its expression is downregulated by insulin in the fed state and upregulated by fasting or during adipocyte differentiation (32–36, 39).

PNPLA2 catalyses the hydrolysis of triacylglycerol on the surface of lipid droplets at the *sn*-1 and *sn*-2 position, which is the first step in triacylglycerol degradation in the fasted state to obtain energy, a process called lipolysis (Figure 3; 34, 35, 39–42). Triacylglycerol is hydrolysed to diacylglycerol and non-esterified fatty acid, which is further degraded by hormone-sensitive lipase to monoacylglycerol and non-esterified fatty acid. Finally, monoglyceride lipase hydrolyses monoacylglycerol to glycerol and non-esterified fatty acid. As a result, non-esterified fatty acids are mobilised in tissues that require energy, and glycerol enters gluconeogenesis.

Besides triacylglycerol, PNPLA2 hydrolyses retinyl esters (RE) (37, 43) and to a lower extent acts as phospholipase A2 and transacylase (41). A recent study (44) has reported that PNPLA2 utilises its transacylase activity in the synthesis of fatty acid esters of hydroxy fatty acids, a subclass of fatty acids with potential anti-inflammatory and anti-diabetic roles, by transferring an acyl chain from triacylglycerol or diacylglycerol to hydroxy fatty acids.

Some studies (33, 35, 38, 41, 45) indicate that, like PNPLA1, PNPLA2 is activated and translocated to lipid droplets by interaction with CGI-58. Namely, PNPLA2 interacts with CGI-58 through the *N*-terminal domain, once it dissociates from perilipin-1, a protein coating and protecting lipid droplets from lipolysis. When energy is needed, β-adrenergic stimulation activates protein kinase A which phosphorylates perilipin-1 on several amino acid residues and releases CGI-58, which then interacts with PNPLA2, recruits it to the lipid droplet, and stimulates lipolysis (46). Interestingly, in the absence of CGI-58, PNPLA2 hydrolyses triacylglycerol species only at the *sn*-2 position, whilst in its presence, the hydrolysis occurs at both the *sn*-1 and *sn*-2 position (47).

Several *in vitro* studies have discovered that the enzymatic activity of PNPLA2 is inhibited by the interaction with the G0/G1 switch protein 2 (G0G2) (33, 40, 41, 48, 49), the hypoxia-inducible lipid droplet-associated protein (HILPDA) through the *N*-terminal domain (38, 46), and the long-chain acyl-CoAs (49). The latter could be a feedback mechanism to protect cells from non-esterified fatty acid accumulation, which is toxic to cells.

Several *in vitro* and *in situ* studies (36, 40, 45, 50) suggest that the enzymatic activity of PNPLA2 in tissues with high fatty acid oxidation, such as cardiac and skeletal muscle, is regulated by interaction with perilipin-5, another protein that coats and protects lipid droplets. It is proposed that, in the fasted state, perilipin-5 directly binds to PNPLA2 and CGI-58 and then promotes their interaction with lipid droplets and eventually lipolysis.

Currently we know of six mutations occurring in the C-terminal patatin domain or in the *N*-terminal domain that encode an aberrant PNPLA2 associated with the development of a rare autosomal recessive disorder called neutral lipid storage disease (NLSD) (35, 41, 45). It is caused by the accumulation of triacylglycerols in almost

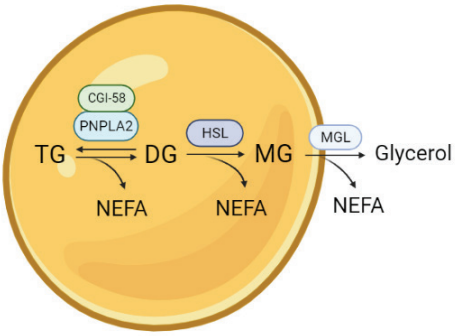

Figure 3 Simplified representation of lipolysis on lipid droplets (created courtesy of Biorender.com). PNPLA2, activated by CGI-58, hydrolyses triacylglycerol (TG) generating diacylglycerol (DG) and non-esterified fatty acid (NEFA). Then, hormone-sensitive lipase (HSL) hydrolases diacylglycerol (DG) generating monoacylglycerol (MG) and non-esterified fatty acid (NEFA) and finally, monoacylglyceride lipase (MGL) hydrolyses monoacylglycerol (MG) generating non-esterified fatty acid (NEFA) and glycerol

every tissue and manifests as cardiac and skeletal myopathy and liver steatosis (35, 41).

Considering that abnormal lipid metabolism with high fatty acid release contributes to various metabolic syndromes (51) and cancer (52), several *in vivo* studies (51, 53) have shown that PNPLA2 inhibition slows down the onset of high-fat diet-induced insulin resistance and the progression of non-alcoholic fatty liver disease (NAFLD) to a more severe stage such as steatohepatitis. *In vitro*, PNPLA2 enhances lipolysis and therefore promotes colorectal (54) and hepatocellular cancer (55, 56). Its inhibition was reported to slow down breast cancer metastasis into the lungs (57, 58) and that PNPLA2 inhibitors such as atglistatin reduce tumour growth *in vivo* (52). However, the exact mechanisms of PNPLA2 action in terms of cancer development are yet to be elucidated.

PNPLA3 (EC 2.3.1.51)

With its 481 amino acids and the *N*- and C-terminal domain PNPLA3 (aka adiponutrin) is highly homologous to PNPLA2 (5, 15, 31, 48, 59, 60). Like PNPLA2, its patatin domain lies in the same region of amino acid residues (at positions 10–179) with the catalytic dyad Ser47-Asp166 (5). PNPLA3 is located in the cytoplasm and is recruited to lipid droplets in a way similar to PNPLA2 (15, 60–63). In humans, PNPLA3 is highly expressed in hepatocytes and hepatic stellate cells (60) and to a lower extent in the adipose tissue, kidneys, brain, and skin (38, 63).

Unlike PNPLA2, PNPLA3 is nutritionally and hormonally regulated in the opposite direction (48, 60). In the fasted state, PNPLA2 is upregulated and PNPLA3 downregulated, which suggests that PNPLA3 plays a role in lipogenesis and lipid droplet restoration (31, 48, 60). In the fed state, PNPLA3 behaves much

like PNPLA2, as it also binds to lipid droplets through interaction with CGI-58 (62, 63) and, in fact, competes with PNPLA2 for this activator enzyme (60). However, the physiological function of PNPLA3 is yet to be defined.

PNPLA3 can act as triacylglycerol hydrolase at the *sn*-2 position of the glycerol backbone and as thioesterase and acyltransferase on phospholipids (15, 59−61, 63, 64). This enzyme also hydrolases retinyl esters in hepatic stellate cells (60, 64) and catalyses conversion of lysophosphatidic acid to phosphatidic acid (59). In addition, PNPLA3 shows specific acylglycerol transacylase activity for lysophosphatidic acid and long-chain acyl-CoAs, provided that the substrate acyl group has at least 16 or more carbon atoms (59).

PNPLA3 attracted attention with the discovery of its gene polymorphism in which cytosine is replaced with guanine and consequently isoleucine with methionine at the position 148 in the amino acid sequence (I148M) (59, 65). This mutation is associated with the development of various liver diseases such as NAFLD, non-alcoholic steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (59, 66) due to extensive accumulation of triacylglycerol in the liver.

On a molecular level, the expression of PNPLA3 I148M seems to be regulated by the pro-inflammatory transcription factor NF-kB, which can lead to high TNF-α levels and steatohepatitis (67). One study (68) found that the increased activity of the IL6/STAT3 signalling pathway enhances susceptibility to NAFLD development mediated by PNPLA3 I148M. Several other *in vitro* studies (61−63) have shown that triacylglycerol hydrolysis by the mutated PNPLA3 (I148M) variant drops by 80–90 %, suggesting that methionine blocks the active site to its substrates (61−63). One research (59) has shown that the I148M variant has higher lysophosphatidic acid transacylase activity, while other studies (61, 62, 65, 69) report that it highly accumulates on lipid droplets, rendering them larger than normal. Abnormal accumulation of I148M impairs regular lipid metabolism and even disrupts the regular activity of PNPLA2, which may lead to inflammation and fibrosis (63, 70). Furthermore, the mutated PNPLA3 I148M variant interacts with CGI-58 more strongly than the non-mutated protein (60, 62, 63, 71, 72). More recent research has shown that I148M is resistant to ubiquitination or autophagy, which results in its accumulation on lipid droplets (62, 63, 66, 69, 71).

PNPLA4 (EC 3.1.1.3)

With only 253 amino acids PNPLA4 (aka GS2) is the smallest PNPLA enzyme (5, 73), located in the cytoplasm in a membrane-free form, since it only contains the *N*-terminal domain with the active site (20). The *N*-terminal domain accommodates the patatin domain with catalytic dyad Ser43-Asp163 (with the respective lipase motifs GASAG and DGG) (5). PNPLA4 is expressed in almost every tissue of the body including the adipose tissue, liver, muscles, kidneys, lungs, placenta, and brain (31, 65).

In vitro studies show that PNPLA4 acts as triacylglycerol and retinyl ester hydrolase (73, 75) and transacylase for a variety of acyl-donors (4, 40, 76, 77). Both retinyl ester hydrolase in the skin and transacylase activities are inversely regulated by pH (74, 75). Hydrolysis is stronger at neutral pH and transacylase activity at acidic pH (73). One study (75) has identified the 47 kDa tail-interacting protein (TIP47) as its inhibitor, suggesting that TIP47 regulates retinyl ester levels and thereby participates in the regulation of keratinocyte differentiation.

PNPLA5 (EC 3.1.1.3)

PNPLA5 (aka GS2-like) has 429 amino acids (5, 20) and a structure similar to the previously described PNPLAs with the *N*-terminal domain (with the active site) and the C-terminal domain (20). The patatin domain is located in the residues 12–181 within the *N*-terminal domain and accommodates the catalytic dyad Ser49-Asp168 (with the respective lipase motifs GSSSG and DGA) (5). Experiments show that PNPLA5 binds to lipid droplets in the cell through the conserved arginine or positively charged amino acids located on the positions 358–361 in the amino acid sequence (20, 78). In humans, PNPLA5 is expressed in almost every tissue, but peaks in the brain and pituitary gland (30). Similar to PNPLA3, its expression is low in the fasted state and high during adipocyte differentiation (31, 78).

Like other PNPLAs, PNPLA5 displays triacylglycerol hydrolase activity (78) and is essential for an optimal initiation of autophagy, as illustrated in Figure 4 (79−81). The role of PNPLA5 is to help lipid droplets and triacylglycerols form an autophagosome bilayer membrane (79, 80, 82).

PNPLA6 (EC 3.1.1.5)

PNPLA6, also known as neuropathy target esterase (NTE), is a much larger enzyme than those previously described, as it contains 1327 amino acids (5, 83). Its structure is familiar – *N*-terminal and C-terminal domain (83−86) – but, unlike ADPN enzymes, it is the C-terminal domain that contains the active patatin domain with catalytic dyad Ser1014-Asp1134 with amino acid residues 981–1147 (and respective lipase motifs GTSIG and DGG) (5). It is catalytically competent alone (84, 87). The *N*-terminal domain contains a transmembrane segment and regulator segment with three cyclic nucleotide-binding sites (84, 87). However, there is no evidence that cyclic nucleotides directly bind to PNPLA6 (85). Transmembrane segment is located in residues 60–80, and cyclic nucleotide binding sites 1, 2, and 3 are located in residues 195–322, 511–623, and 629–749, respectively (5).

PNPLA6 can bind to the endoplasmic reticulum and lipid droplets (83). The *N*-terminus of the enzyme is oriented towards the lumen of endoplasmic reticulum, and the rest of the enzyme (the regulator segment and C-terminal domain) is oriented towards

the cytoplasm (87). Optimal binding with the endoplasmic reticulum requires the transmembrane segment and the whole regulatory segment (87). In addition, the C-terminal domain alone shows high affinity for lipid droplets and its binding is independent of the enzyme's catalytic activity (87).

PNPLA6 is highly expressed in the brain and lymphocytes and to a lower extent in the spinal cord, liver, kidneys, placenta, and spleen (16, 85, 87, 88).

As a phospholipase B enzyme it catalyses the hydrolysis of acyl substituents from the *sn*-1 and *sn*-2 positions of phospholipids (84, 85, 89), preferably lysophosphatidylcholine and phosphatidylcholine (88–91), but also acetylcholine and monoacylglycerol (85).

The physiological role of PNPLA6 is not fully understood, but it may be important for the homeostasis and fluidity of cell membranes (88). PNPLA6 is also essential for embryonal development, as demonstrated in PNPLA6 knock-out mice, whose placental failure and impaired vasculogenesis led to the death of their embryos on gestation day 8 (84, 88, 92). Low expression of PNPLA6 was also reported to affect the development of nervous, vascular, and respiratory systems in embryos (88). In addition, PNPLA6 might be involved in cell differentiation (92).

PNPLA6 was discovered more than 50 years ago as a target of highly toxic organophosphate compounds such as nerve agents and pesticides (83, 88). Its inhibition by organophosphates (Figure 5) leads to the development of the so-called organophosphate-induced delayed neuropathy (OPIDN) syndrome, which is characterised by weakness, loss of coordination, and muscle tingling and cramps (usually in the lower limbs) as a result of long motor axon degeneration in the spinal cord and peripheral nervous system (83, 86, 88, 89). Research has shown that at least 70 % of PNPLA6 has to be inhibited in the nervous system for the OPIDN syndrome to develop (83, 84, 88).

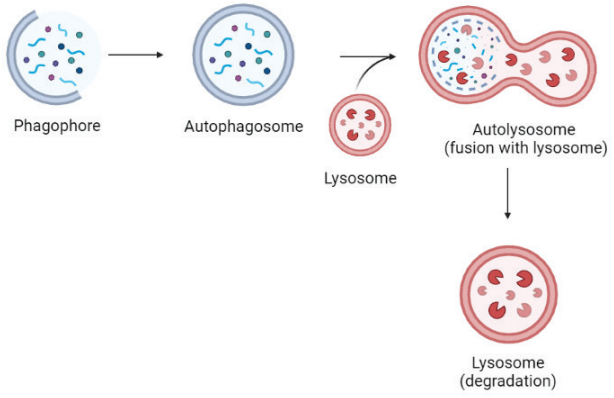

Figure 4 Simplified representation of autophagy (created courtesy of Biorender.com). It starts with the formation of a phagophore which develops into an autophagosome. Autophagosome fuses with a lysosome and degrades it. Blue and purple circles and curved blue dashes in represent proteins and cytoplasmic content that will be eventually degrade in the lysosome. Red and pink Pac-Man-like structures represent hydrolytic enzymes that degrade the autophagosome content

Inadequate PNPLA6 expression can also be the consequence of the so-called "loss-of-function" mutations, which have been associated with diseases of motor neurons and hereditary spastic paraplegia 39 (SPG39) manifested by muscle weakness and paralysis as result of long axon degeneration in the spinal cord (16, 87, 91). In addition, *PNPLA6* gene mutations can cause autosomal recessive disorders such as Boucher-Neuhauser syndrome, Gordon-Holmes syndrome, Oliver-McFarlane syndrome, and Laurence-Moon syndrome, characterised by movement disorders and mental disabilities (83, 84, 93, 94).

PNPLA7 (EC 3.1.1.5)

PNPLA7 is another NTE-related esterase or NRE containing 1317 amino acids (4, 5). Similar to PNPLA6, this enzyme contains the C-terminal domain with the active site and the *N*-terminal domain with the transmembrane and regulator segment containing three cyclic nucleotide binding sites (95−97). The patatin domain is located in the C-terminal domain in amino acid residues 928–1094 and accommodates the catalytic dyad Ser961-Asp1081 (with the respective lipase motifs GTSIG and DGG, like PNPLA6) (5). The transmembrane segment is located in a similar region of the amino acid sequence as in PNPLA6 (residues 13–33), and the cyclic nucleotide binding sites 1, 2 and 3 are located in residues 145–272, 458–563, and 591–696, respectively (5).

As in PNPLA6, the *N*-terminus is oriented towards the lumen of the endoplasmic reticulum, and the rest of the enzyme is oriented towards the cytosol (96). PNPLA7 binds to the endoplasmic reticulum and lipid droplets in the cell (96, 98). It anchors to the endoplasmic reticulum through the transmembrane segment but also needs the regulator segment to bind optimally (95, 96). It seems to bind to lipid droplets through its C-terminal domain non-enzymatically (96, 99).

In vivo studies (97, 98) show that PNPLA7 is highly expressed in the testes and tissues targeted by insulin, such as skeletal muscles, cardiac muscle, adipose tissue. In mice (98), its mRNA is upregulated in the fasted state and downregulated in the fed state in the testes, skeletal and cardiac muscle, and brown and adipose tissue. Furthermore, it is suppressed by insulin in the 3T3-L1 adipocytes. A recent study with cultured human myotubes (100) has shown that insulin suppresses mRNA expression in physiological conditions and dibutyryl-cAMP in the fed state and that PNPLA7 protein levels inversely correlate with glucose concentrations. All this suggests that PNPLA7 is regulated by the nutritional status and plays a role in energy metabolism.

As a lysophospholipase PNPLA7 prefers unsaturated phospholipids such as lysophosphatidylcholine, lysophosphatidic acid, and phosphatidylethanolamine, which it hydrolyses to glycerol-3-phosphocholine and a free fatty acid. In contrast, it does not

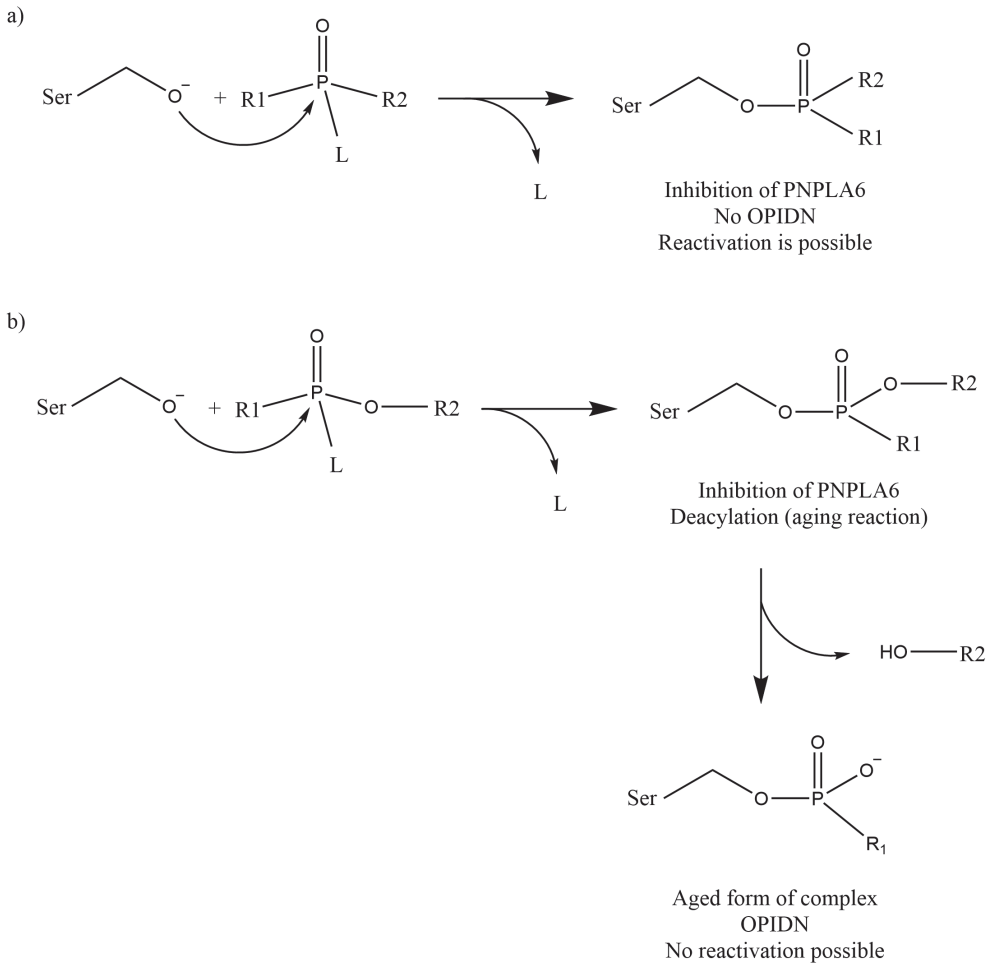

Figure 5 Simplified scheme of PNPLA6 inhibition with organophosphate compounds (OP). It starts with a nucleophilic attack of the hydroxyl group of the serine on the phosphorus atom from the OP, which leads to the formation of a tetrahedral intermediate. R1, R2 – acyl groups, L – leaving group. a) Reaction with OPs which does not undergo aging and does not cause OPIDN and the enzyme can be reactivated. b) Reaction with OPs containing the P-O-R or P-NH-R bond that undergoes aging, in which the R-group spontaneously leaves the intermediate. As a result, the phosphate group is negatively charged and stays covalently bound to the serine in the active site. The enzyme cannot be reactivated because of the negative charge. The negatively charged phosphate group is stabilised through hydrogen bonds in the oxyanion hole. OP compounds that undergo the aging reaction can cause neuropathy and OPIDN

significantly hydrolyse phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine and does not hydrolyse triacylglycerols, monoacylglycerols, cholesteryl esters, and retinyl esters *in vitro* (96, 98).

In one of our studies (100), we found that knocking down the mRNA of *PNPLA7* in cultured human myoblasts significantly reduces the α1-subunit of Na^+K^+-ATPase, acetyl-CoA carboxylase, phosphorylated forms of the 70 kDa ribosomal S6 kinase, and ribosomal protein S6, which suggests that PNPLA7 is important for the function of human myoblasts in a number of aspects (100). Several studies (101−103) in which *PNPLA7* was knocked down have confirmed the biological importance of PNPLA7 in energy metabolism and its role in the development of metabolic disorders. There are reports of myopathic changes, including inflammation and degeneration of myocytes, exocrine cells, hepatocytes, and adipocytes (101). Moreover, the knockdown of *PNPLA7* in mice hepatocytes has been reported to reduce the secretion of very low-density-lipoproteins and increase the accumulation of triacylglycerols as a result of increased ubiquitylation of apolipoprotein E. PNPLA7 seems to interact with apolipoprotein E, presumably at the endoplasmic reticulum, and decelerates its degradation, which in turn stimulates the secretion of very low-density lipoproteins from hepatocytes (102). A more recent study (103) has shown that both *PNPLA7* and *PNPLA8* knock-out mice have altered hepatic catabolism of phosphatidylcholine and display systemic abnormalities consistent with methionine deficiency. All this suggests that PNPLA7 and PNPLA8 play a key role in generating glycerol-3-phosphocholine and choline, whose methyl groups are utilised in the methionine cycle.

PNPLA8 (EC 3.1.1.5)

PNPLA8, also known as iPLAγ, is a membrane protein containing 782 amino acids and consisting of the C-terminal and *N*-terminal domains, just like the other PNPLAs. The patatin domain with the catalytic dyad Ser483-Asp627 is located in the C-terminal domain in the amino acid residues 445–650 and accommodates the catalytic dyad Ser483-Asp627 (with the respective lipase motifs GVSTG and DGG) (5, 10, 104, 105). The *N*-terminal domain is rich in serine and threonine, potential sites for phosphorylation by protein kinases (104, 105). Depending on

localisation sequence, PNPLA8 can bind to peroxisome or mitochondrial membranes (105–109) and to the endoplasmic reticulum and autophagosome (110).

PNPLA8 is highly expressed in the myocardium and to a lower extent in the placenta, skeletal muscles, brain, liver, pancreas, and lungs (4, 10, 104, 106).

It mainly acts as phospholipase A1 and has low lysophospholipase activity (108). However, with phospholipid substrates esterified with the polysaturated fatty acid chain at the *sn*-2 position such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, PNPLA8 readily acts as phospholipase A1 to yield 2-arachidonoyl-lysophosphatidylcholine (2-AA-LPC) (107, 108, 111, 112). 2-AA-LPC is a lipid naturally present in the human myocardium and is central for cardiac metabolic signalisation. It can further get metabolised to endocannabinoids and be involved in eicosanoid signalisation (106, 108, 111−113).

In addition, PNPLA8 hydrolyses cardiolipin, a phospholipid solely present in the mitochondrial membrane and essential for normal function of mitochondria. (111, 113, 114). Without this hydrolysis, as shown in *PNPLA8* knock-out mice (111), cardiolipin levels increase, which results in inefficient electron coupling, reduced mitochondrial bioenergy efficiency, and altered cellular signalling.

PNPLA8 also supplies lysophosphatidylcholine to PNPLA7, and both enzymes have a key role in hepatic phosphatidylcholine metabolism supplying methyl groups in the methionine cycle (103).

Even though we do not have a full grasp of the physiological and regulatory role of PNPLA8, various studies suggest that PNPLA8 maintains phospholipid homeostasis, optimal function of mitochondria, metabolic signalling, and the opening of mitochondrial permeability transition pore (mPTP) (113–116). Loss of PNPLA8 activity, through knock-out or genetic ablation, can result in impaired mitochondrial function, generation of reactive oxygen species, increased lipid peroxidation, reduced adenosine triphosphate (ATP) and glutathione levels in the skeletal muscle and liver, and ultimately apoptosis (103, 113, 117−121). One study (115) suggests that PNPLA8 can protect the cell against apoptosis by promoting the repair of peroxidised cardiolipin in mitochondria (113). It also seems to play an important role in mPTP opening, judging by studies reporting inadequate mPTP opening in PNPLA8 knock-out mice (121−123).

PNPLA9 (EC 3.1.1.4)

PNPLA9, also known as PLA2G6 and iPLAβ, contains 806 amino acids and is the only PNPLA enzyme with solved crystal structure (Figure 6) (5, 17, 124). It consists of the *N*-terminal domain, catalytic domain, and domain with ankyrin repeats (5, 17). The patatin domain is in the catalytic domain among amino acid residues 481–665, which accommodate the active site Ser519-Asp652 and the oxyanion hole, a motif rich in glycine (17, 124, 125). The active site is positioned in a wide cavity to accommodate phospholipids with long unsaturated fatty acid chains (17, 124). Unlike the rest of PNPLA enzymes, PNPLA9 lacks transmembrane domains but is rich in motifs that can interact with other proteins (17). One such motif (and PNPLA9 has nine) is ankyrin repeat, which consists of 33 amino acid residues ready to interact with related receptor proteins (17, 124, 126, 127).

Judging by its crystal structure, PNPLA9 is a stable dimer in its active form (17, 124, 128). Namely, active sites are located in the close vicinity to each other, and the disruption of the dimer inactivates the enzyme (17, 124).

PNPLA9 interacts with ATP, Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase), and calnexin, a chaperon protein located in the endoplasmic reticulum (10, 17, 107, 124, 126, 129). CaM kinase has been reported to inhibit the catalytic activity of PNPLA9 in the presence of calcium by stabilising the closed dimer conformation (17, 107, 124).

PNPLA9 is expressed mostly in the cytoplasm and can bind to various cell organelles such as the cell membrane, mitochondria, endoplasmic reticulum, Golgi apparatus, and the nuclear envelope (4, 124, 126, 128−132). However, we still do not understand the mechanisms of its binding with the membranes of these cell organelles (17).

In vitro, PNPLA9 catalyses the hydrolysis of fatty acids at the *sn*-2 position of glycerophospholipids, preferably plasmalogens (4, 17, 107, 124, 129–131). The products of such hydrolysis are free fatty acids and lysophospholipids, secondary messengers in various signalling pathways (109, 126, 130, 132, 133). PNPLA9 also shows transacylase and thioesterase activity *in vitro* (10, 130).

PNPLA9 is involved in numerous important biological processes, including cell growth and migration, remodelling of cell membranes, autophagy, release of arachidonic acid induced by agonists, insulin secretion, bone formation, contraction and relaxation of blood vessels, and apoptosis, but its exact role in these processes is not fully understood (4, 10, 124, 127, 130, 131, 133, 134). PNPLA9 knock-out mice have been reported for neurological damage, because of disrupted axon membrane homeostasis and accumulation of ubiquitinated proteins (4, 10, 130, 132, 134), and for inner mitochondrial and presynaptic membrane disruption and impaired Ca^{2+} uptake by astrocytes (10, 134).

Impaired PNPLA9 activity is associated with cardiovascular diseases, tumours, diabetes, muscle dystrophy, non-alcoholic steatohepatitis, antiviral response (10, 17, 129, 135–140), and the development of various neurological and neurodegenerative syndromes (106, 132, 134, 137, 141). All diseases caused by *PNPLA9* mutations or its irregular activity are collectively referred to as PLAN or PLA2G6-associated neurodegeneration, which is characterised by the formation of spheroids in both the central and peripheral nervous system as a result of the accumulation of membranes, organelles, and misfolded and ubiquitinated proteins in neurons (125, 135, 141). Such mutations are found in each PNPLA9 domain and each mutation can have a different effect on the catalytic activity, regulation, and potential macromolecular interactions (10, 17, 124),

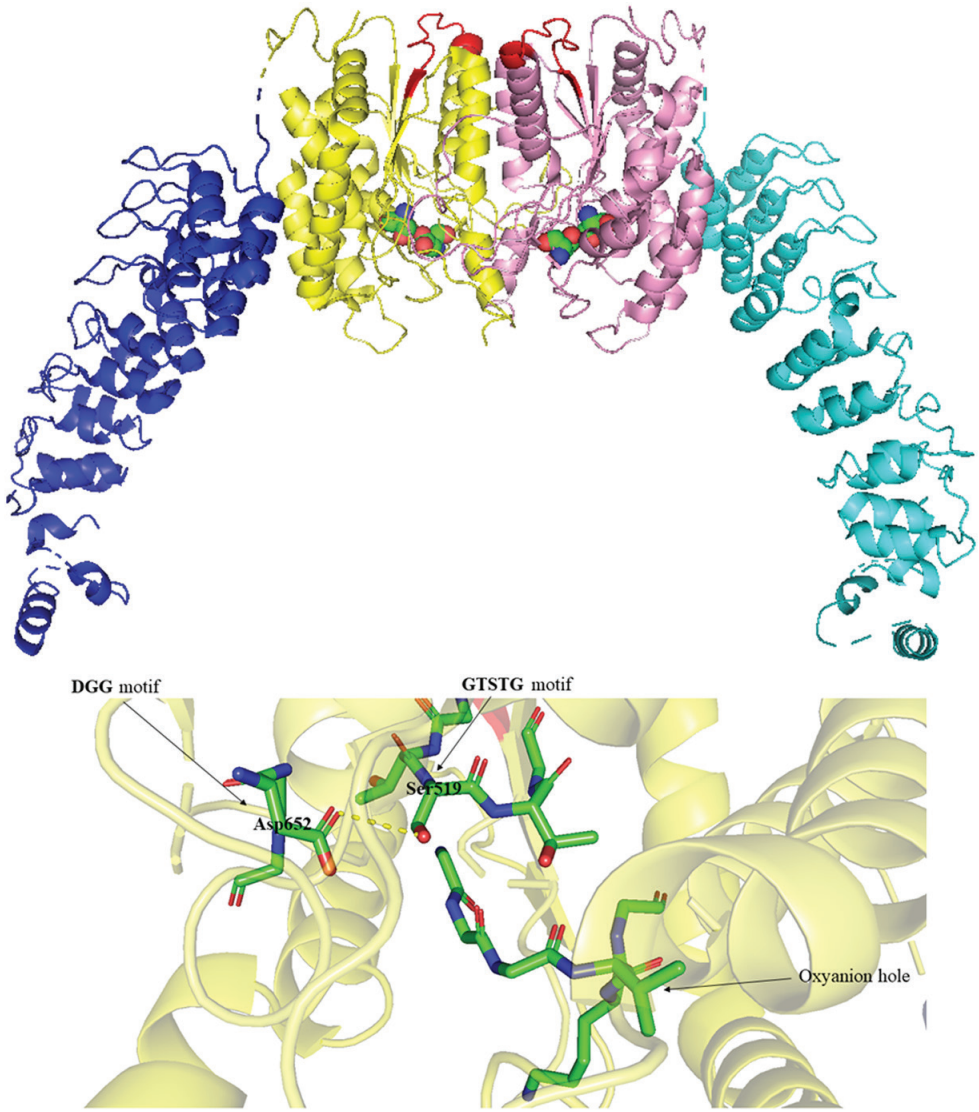

Figure 6 3D illustration of the PNPLA9 dimer (PDB: 6AUN) (created with PyMol). Top: catalytic domains of monomer one and monomer two are coloured yellow and pink, respectively. Active sites represented by spheres and sticks. Ankyrin repeats of monomer one and monomer two are coloured dark and light blue, respectively. The binding site of CaM kinase is in red. Bottom: the active site of PNPLA9 contains the catalytic dyad serine-aspartic acid, with the catalytic serine situated on a nucleophilic elbow following a β-sheet and preceding an α-helix. Serine is part of the lipase Gly-Thr-Ser-Thr-Gly motif and aspartic acid is a part of the Asp-Gly-Gly motif. Close to the active site is an oxyanion hole characterised by the Gly-Gly-Gly-Arg motif, whose function is to stabilise the transition state. Yellow dashed line represents a hydrogen bond formed between serine and aspartic acid

yet all ultimately lead to the development of neurodegenerative diseases characterised by iron accumulation such as Alzheimer's disease, Parkinson's disease, Karak syndrome, infantile neuroaxonal dystrophy (INAD), and neurodegeneration with brain iron accumulation (NBIA) (4, 10, 129, 132, 135). Interestingly, PNPLA9 mutation can also be associated with Alzheimer's and Parkinson's disease without iron accumulation (10).

Future PNPLA-related research and possible challenges

The PNPLA family of enzymes with its diverse members, from the smallest containing only 253 to the largest with more than 1360 amino acids, has a potential to become the new target for drug development, as it has an essential role in lipid remodelling. Each member of this family contains a patatin domain with the Ser-Asp active site and an oxyanion hole, both enabling its specific biochemical activity. Numerous knock-out, gain-of-function, and loss-of-function research models evidence its importance in various biological processes, such cell membrane homeostasis and integrity, cell growth, signalling, cell death, and the metabolism of lipids like triacylglycerol, phospholipids, ceramides, and retinyl esters. Besides gene loss, research has shown a connection between specific mutations and irregular catalytic activity of certain PNPLA members and the development of various diseases.

PNPLA research is yet to gather information about the basic kinetic properties, substrate preferences, and the mechanisms of regulation at the gene and protein level to help design prospective anti-PNPLA acting drugs (1, 12). One direction in kinetic research, for example, could be to use tissue or cell lysates with overexpressed enzyme and a substrate specific enough to determine and distinguish PNPLA catalytic activity (16, 142, 143). However, a better approach would be to purify the active form of these enzymes for kinetic characterisation. Yet, these ideas may be difficult with some

membrane-bound PNPLA members, like PNPLA6 and PNPLA7, not only because of possible aggregation as insoluble fractions, but also because their overexpression is toxic to cells because of their lysophospholipid activity (4). In our research we have tried to express truncated PNPLA7 in *Escherichia coli* and purify it for kinetic characterisation, but most of the enzyme remained in the inclusion bodies as insoluble fraction (unpublished results). Even so, getting the pure enzyme is the necessary first step to determine its crystal structure (17). Once it is known (like it is for PNPLA9), it would be possible to determine the function of other domains besides patatin, possible interactions with other proteins or molecules, and possible conformation changes. Moreover, other PNPLA enzymes might be functional as multimers, like PNPLA9 or patatin itself (17, 143, 144).

It is important to continue research of this family of enzymes to better understand their activity, biological role, and role in the development of diseases, which can then be targeted by drugs. The PNPLA family of enzymes will receive more attention in the future regardless of any challenges that may arise.

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PNPLA porodica enzima – karakterizacija i biološka uloga

Ovaj revijalni rad donosi pregled dosadašnjih spoznaja o porodici PNPLA (engl. *patatin-like phospholipase domain-containing proteins*) ljudskih enzima. Iako ovu porodicu čini samo 9 članova, najmanji sadrži 253 aminokiseline, a najveći više od 1360 aminokiselina, fiziološka uloga i mehanizam katalitičke aktivnosti nisu do sada potpuno razriješeni niti za jednoga. Međutim, rezultati brojnih tzv. *knock-out*, *gain-of-function* i *loss-of-function* modela istraživanja upućuju na važnu ulogu ovih enzima u mnogim biološkim procesima, uključujući održavanje homeostaze i integriteta stanične membrane, rast stanice, staničnu signalizaciju, staničnu smrt i metabolizam lipida kao triacilglicerola, fosfolipida, ceramida i retinil estera. Također, rezultati istraživanja upozoravaju na povezanost mutacija i nepravilne aktivnosti pojedinih članova s razvojem raznih bolesti. Radi boljeg razumijevanja PNPLA porodice enzima i naglaska na njihov potencijal kao mete razvoja novih lijekova, donosimo sveobuhvatni pregled do sada poznatih spoznaja koje uključuju strukturu, lokalizaciju u stanici, distribuciju u tkivima, specifičnost prema supstratima i potencijalnu fiziološku ulogu.

KLJUČNE RIJEČI: fosfolipaze; katalitička dijada; lipidne kapljice; patatin; serinske hidrolaze